To my parents...

The Influence of Magnesium Ions on the Growth and Metabolism of *Saccharomyces cerevisiae.*

A. I. Maynard B.Sc. (Hons).

A thesis submitted in partial fulfilment of the requirements of Dundee Institute of Technology for the award of the degree of Doctor of Philosophy.

March 1993

Dundee Institute of Technology
in collaboration with
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Abbreviations

Abbreviations, acronyms, conventional and idiosyncratic shorthand used throughout the main body of the text are expanded below. The convention of writing certain words and phrases in full upon their first appearance and then abbreviating upon subsequent usage is adhered to throughout the thesis. Examples include organisms' names and media acronyms.

ADP	=	Adenosine diphosphate
AMP	=	Adenosine monophosphate
ATP	=	Adenosine triphosphate
a.k.a.	=	also known as
B.P. Ltd.	=	British Petroleum Limited
Ed.	=	Edited
EMP	=	Embden-Meyerhof-Parnas
FDA	=	Food and Drugs Administration
FID	=	Flame Ionization Detector
GC	=	Gas Chromatograph(y)
g	=	grams
g/L	=	grams per Litre
HPLC	=	High Pressure Liquid Chromatograph(y)
Kg	=	Kilogram
K_m	=	Michaelis-Menton co-efficient
L	=	Litre(s)
L.S.L. Ltd.	=	Life Science Laboratories Limited
mA	=	milliamps
MAFF	=	Ministry of Agriculture, Food and Fisheries
ml	=	millilitres
µg/ml	=	microgrammes per millilitre
µM	=	micro-Molar

μ_{max}	=	specific maximum growth rate
NAD	=	Nicotinamide Adenine Dinucleotide
nM	=	nano-Metres
^{o}C	=	degrees Centigrade
PGAL	=	Phosphoglyceraldehyde
ppm	=	parts per million
psi	=	pounds per square inch
rpm	=	revolutions per minute
spp	=	species
TCA	=	Tri-carboxylic Acid
YEPD	=	Yeast Extract-Peptone Dextrose

Abstract

The Influence of Magnesium Ions on the Growth and Metabolism of *Saccharomyces cerevisiae:* A. I. Maynard.

The growth response of batch cultures of *Saccharomyces cerevisiae* to magnesium-limitation, under conditions of micro-aerophillic glucose-repression, was shown to be hyperbolic, thus indicating a Monod relationship. The maximum growth rate was found to be $0.20hrs^{-1}$ whilst K_S was equal to 36μM and the yield co-efficient at $^1/_2\mu_{max}$ equal to 1.9grams of cells formed per milligram of magnesium removed from the medium. The suggested downward revision of the yeast's exogenous magnesium requirements from 2-4mM (Jones and Greenfield, 1984) to 0.5mM (Jones, 1986) is confirmed, with a specific level of 0.65mM for a minimal medium at 2.5%w/v glucose being recommended. A molasses complex medium was found to contain levels in excess of 40mM although the possibility of magnesium-limitation within an industrial context still exists.

Magnesium uptake patterns were established over a range of limiting (11-650μM) and non-limiting (650-4000μM) concentrations of exogenous magnesium. The uptake patterns obtained corresponded to the changes in the metabolic activities of the population further indicating the highly regulated nature of the ion within the yeast cell (Jones and Greenfield, 1984). The patterns of uptake and release suggests a means of 'priming' the cells to establish control over the fermentative capacity of the organism through the timing of the inoculum transfer.

The investigation of the physiological states of *S. cerevisiae* over a range of growth rates using a magnesium-limited chemostat was carried out with the results agreeing in the main with the trends established in the batch cultures. At growth rates lower than μ_{max}, the cells reveal a physiological state characteristic of non-carbon-limited growth. This involves an increase in the biomass yield from magnesium and alterations in the relative contributions of fermentation and respiration to the overall respiro-fermentative activity as the growth rate increases towards μ_{max}. At growth rates greater than μ_{max}, the cells exhibit pseudo-hyphal growth consistent with the involvement of magnesium in cell morphology.

Studies on the metabolic behaviour of *S. cerevisiae* when released from magnesium-limitation within a chemostat have been carried out and indicate that magnesium-limitation is primarily exerting its effect within the cell division cycle.

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CHAPTER 1: INTRODUCTION

1.1. Industrial and Laboratory Aspects of Yeast

As industrial organisms, yeast are of economic importance, and their ubiquitous presence in the baking, wine, beer and spirit industries is well documented. As Stewart (1988) states: "Brewing, viticulture, enology and distilled beverages are biotechnological industries that make a significant contribution to the economy of most countries around the world". *Saccharomyces cerevisiae* is the yeast species of greatest quantitative significance (Evans, 1990), although other species such as *Kluyveromyces*, *Candida* and *Pichia* play an ever-increasing role in industrial processes (Spencer & Spencer, 1990). Nevertheless, the number of yeast species of economic importance is relatively low compared to the total number described (Lodder, 1970; cited by Peppler, 1979).

Yeast substrates and products are as varied as the species and include: *Kluyveromyces* being grown on whey permeate to secrete invertase; *Candida* and *Pichia* grown on ethanol and methanol respectively, to form single cell protein; and *Candida* grown on n-alkanes for the production of citric acid. Other areas currently being investigated include: the use of *Hansenula* spp. to produce steroid precursors; *Lipomyces* spp. to produce lipids; the synthesis of organic acids using such species as *Torulopsis*, *Candida*, and *Saccharomycopsis*; and the potential use of yeast in pollution control. These diverse attributes are covered in detail by Burden and Eveleigh (1990), who also comment that few of the ideas for the biotechnological use of non-*Saccharomyces* species achieve commercial status despite extensive research.

Many mycelial fungi are proving useful at secreting proteins and secondary metabolites of commercial interest (Upshall & McKnight, 1987) but in spite of the lack of secondary metabolites of immediate commercial interest produced by *S. cerevisiae* (Stewart & Russell, 1986), the range of nutritional and flavouring products derived from the cells themselves is continually expanding (Reed & Nagodawithana, 1991), as is the organism's involvement in the production of non-yeast proteins and peptides such

as the anti-viral protein interferon (Hitzman, Leung, Perry, Kohr, Levine, & Goeddel, 1983), human serum albumin (Molzahn, 1985) and the acid protease chymosin (Smith, Duncan, & Moir, 1985).

Currently, the application of recombinant technology to yeasts has permitted the development of 'novel' yeast products, many of which are at a "near-market" level of completion with several already having achieved FDA-approval (Barr, Gibson, Bathurst, Sabin, Medina-Selby, Coit, & Valenzuela, 1989). A selection of these new products is presented in Table 1.1 below.

Table 1.1: Novel yeast products from the application of recombinant technology.

Product Area	Product	Uses	Level of Completion
Food	Rennin	Cheese production	FDA-approval
Food	Improved Baker's yeast	Glucose de-repression	MAFF-approval
Beverage	Improved Brewer's yeast	Amylolytic activities	Research-stage
Pharmaceutical	Hepatitis-B virus surface-antigen	Hepatitis vaccine	FDA-approval
Pharmaceutical	β-Interferon	Treatment of multiple sclerosis	FDA-approval
Pharmaceutical	Circumsporozoite protein from *Plasmodia vivax*	Malaria vaccine	Clinical trials
Pharmaceutical	HIV envelope (env) genes	Aids vaccine	Clinical trials

Although the expansion of *S. cerevisiae* out of its historic areas of application using genetic transformation techniques may not be ideal for consumer acceptability, especially relating to food, the species does remain an important experimental model (Stewart & Russell, 1986) for the characterization of basic physiological and genetic processes of eukaryotic cells, and for the study of yeast pathogenic behaviour in plants and animals (Russell, Jones, & Stewart, 1987). Whilst research into the metabolic

processes of industrial strains is of importance (Jones & Gadd, 1990; Lievense & Lim, 1982) concerning the efficient conversion of substrate-to-product, it is laboratory strains that have been most fully described (Russell *et al.*, 1987; Stewart & Russell, 1986) in terms of their genetics and biochemistry (Lievense & Lim, 1982), possibly due to ease of accessibility (Fiechter, Fuhrmann, & Kappeli, 1981). This may not accurately anticipate the future needs of biotechnological processes where polyploids will be favoured due to the inherent stability of the multiple-gene structure. However, haploid characteristics that are suitable for laboratory molecular studies, such as the ability to form stable mutants, are not found in industrial strains. These strains also tend to lack mating-type characteristics whilst any spores formed exhibit a low viability (Keiding, 1985; Stewart, 1981). Nevertheless, advances are being made in improving the sporogenic ability of a polyploid brewer's strain (Bilinski, Hatfield, Sobczak, Russell, & Stewart, 1987).

1.2. Yeast Intermediary Metabolism

A more detailed understanding of the intermediary metabolism of *S. cerevisiae* and its interaction with the environment is required to ensure efficient substrate utilization and quality product formation. It is van Dijken, Verduyn, Postma, Weusthuis, van Urk, Visser, & Scheffers's view (1990) that because the effects of environmental factors are species-specific, understanding of the regulation of metabolic pathways, especially regarding the control of industrial cultures, is greatly facilitated by comparative studies on yeasts belonging to "different physiological classes". Other authors likewise advocate the expansion of physiological studies to alternative yeast species as a way of determining metabolic regulatory processes for application to industrial processes (Bruinenburg, 1986; Moulin, Boze, & Galzy, 1984; Fiechter *et al.*, 1981). Whilst strongly supporting these views, this study is concerned with the effect of specific environmental characteristics on the carbon metabolism of an industrial strain of *S. cerevisiae*, although references to work on other species will be made where pertinent. Recent comparative studies include Senac and Hahn-Hagerdal (1990);

McDonald and Tsai (1989) and van Urk, Bruinenberg, Veenhuis, Scheffers, & van Dijken (1989a); and van Urk, Schipper, Breedveld, Mak, Scheffers, & van Dijken (1989b); whilst the metabolism of non-*Saccharomyces* species have been examined by Wijsman, van Dijken, van Kleeff, & Scheffers (1984); Bruinenberg, de Bot, van Dijken, & Scheffers (1983) and Moulin *et al.* (1984).

Sugars, as a ubiquitous source of carbon and energy, have received the most attention regarding their catabolism and its regulation in yeast (van Dijken *et al.*, 1990). The common metabolic means of utilization is through glycolysis (McDonald & Tsai, 1989) from glucose-6-phosphate to pyruvate by way of the Emden-Meyerhof-Parnas (EMP) pathway, although access to and departure from this pathway is dependent on the sugar and the yeast species in question (Gancedo & Serrano, 1989). General schemes of carbon and energy metabolism and their regulation in yeast have been documented and are widely available in the literature (e.g. Entian & Barnett, 1992; Gancedo & Serrano, 1989; Young, 1987; Kappeli, 1986; Lievense & Lim, 1982).

The metabolism of glucose by *S. cerevisiae* enables the cell to grow and reproduce and occurs through a complex network of catabolic and anabolic pathways that inter-links energy production with biosynthesis, whereby ATP is manufactured during the catabolic oxidation of organic molecules, with the breakdown products providing a source of metabolic intermediates for anabolic processes. The interweaving of the two systems and their respective component systems requires highly developed regulatory mechanisms to ensure that the survival requirements of the cell, dictated by the external environment, are constantly and adequately met.

Growth on glucose may proceed by two major routes, fermentation and respiration, although the aerobic utilization of the ethanol produced during fermentation may be considered as a third option open to the cells. Fermentation, which is the only pathway available when oxygen is absent, is also believed (Gancedo & Serrano, 1989) to be the major pathway of glucose catabolism during exponential growth under aerobic conditions due to the repression of the respiratory pathway by the glucose itself (Lagunas, 1979; De Deken, 1966). However, respiration does occur at low glucose

concentrations in the presence of oxygen; a phenomenon employed in the industrial production of yeast using fed-batch techniques, although Quain (1988) argues that there is no evidence that respiration plays a role in normal brewery fermentations. The two pathways can co-exist, a state referred to as "respiro-fermentative metabolism" (Kappeli, 1986), although fermentation is believed to be very active compared to respiration (Lagunas, Dominguez, Busturia, & Saez. 1982; Lagunas, 1979; De Deken, 1966) with their relative magnitudes being determined by the culture conditions (Lagunas *et al.*, 1982). For example, pyruvate dehydrogenase with a high substrate-affinity (K_m 0.1-0.2mM; Keha, Ronft & Kresze, 1982), will constantly channel pyruvate towards respiration until the supply exceeds its capacity, whereupon the low affinity (K_m 3-4mM; Banuelos & Gancedo, 1978) pyruvate decarboxylase will direct the overflow towards fermentation (Holzer, 1961). Such a scenario is envisaged in nitrogen-starved ('resting') cells where inactivated glucose-transport systems lower the glycolytic flux and result in a decrease in fermentative activity but a constant respiratory rate (Lagunas *et al.*, 1982).

The third catabolic option open to yeast cells is the oxidation of the energy-rich ethanol produced during fermentation. This results in a phenomenon known as 'diauxic growth' that requires the presence of oxygen within the culture, and may be viewed as a competitive survival mechanism for the yeast, a perspective shared by Lagunas and Gancedo (1983). Thus, fermentation rapidly converts a substrate (glucose), capable of being utilized by nearly all organisms, to a compound that not only has a greatly reduced number of potential consumers, but is also widely toxic to many micro-organisms.

1.2.1. Fermentation in *S. cerevisiae*

Fermentation is the means by which the cell, in the absence of oxygen, can regenerate the oxidized form of the electron-carrier, NAD, after its reduction during glycolysis to NADH. Yeast cells achieve this by the transfer of the electrons from the NADH to acetaldehyde, derived from the pyruvate, to generate ethanol. ATP is

generated only at a substrate-level within the EMP pathway and not during the production of ethanol. Characteristics of fermentation include decreased levels and altered morphology of mitochondria (Mian, Kuenzi, & Halvorson, 1973; Fiechter, Mian, Ris, & Halvorson, 1972; Polakis, Bartley, & Meek, 1965; cited by Lievense & Lim, 1982) and the reduced activity of the TCA cycle, although it still supplies intermediates for biosynthesis (Schatzmann, 1975; cited by Fiechter *et al.*, 1981). For example, succinate production during fermentation is believed to be the result of an operational TCA cycle (Oura, 1977; cited by Fiechter *et al.*, 1981).

The co-factor NAD functions as an electron-carrier within intermediary metabolism as during fermentation it is reduced to NADH when phosphoglyceraldehyde (PGAL) undergoes substrate-level phosphorylation, with its re-oxidation normally occurring when acetaldehyde is reduced to ethanol. However, the involvement of pyruvate in a functioning TCA cycle, albeit at a reduced level, and the consequent accumulation of reducing power invokes the alternative re-oxidation of NADH during the reduction of dihydroxyacetone phosphate to glycerophosphate (Gancedo, Gancedo, & Sols, 1968; cited by Gancedo & Serrano, 1989), the precursor of glycerol. However, although the cell's redox balance is maintained, the ethanol yield is lowered (Oura, 1977; cited by Gancedo & Serrano, 1989) as the process requires ATP (Gancedo & Serrano, 1989) and leaves energy stored within the glycerol. This alternative means of maintaining redox balance was taken advantage of on an industrial basis during both world wars via 'steered' fermentations whereby the acetaldehyde was artificially removed by complexing with sodium bisulphite, thus enhancing glycerol formation that was then used in the production of explosives (Crueger & Crueger, 1984). The synthesis of other compounds by the cell, such as higher alcohols, also constitutes alternative means of maintaining the redox balance (Stewart & Russell, 1986), providing, as Quain (1988) argues, a "fine-tuning" mechanism for the cell.

1.2.2. Respiration in *S. cerevisiae*

Under aerobic conditions, pyruvate from the EMP pathway is channelled via the pyruvate dehydrogenase multi-enzyme complex, to a highly active TCA cycle, where the transfer of electrons to the co-factors NAD and FAD takes place, providing reducing power to the respiratory chain, and thus enabling oxidative phosphorylation to take place. The high number of co-factors derived from the TCA cycle indicates a more complete breakdown of the substrate than during fermentative activity, and whilst respiration is believed to be easily saturated (Kappeli, 1986), the relative contribution of fermentation can differ markedly according to the environmental conditions (Lagunas *et al.*, 1982).

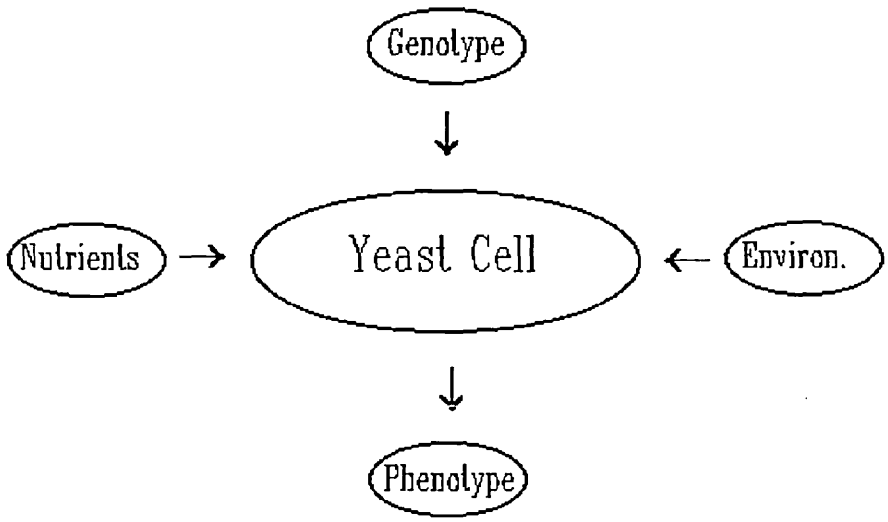
The enzymes pertinent to the TCA cycle and the respiratory chain are mainly localised within the mitochondria (Mian *et al.*, 1973; Ferdouse, Rickard, Moss, & Blanch, 1972; Fiechter *et al.*, 1972; cited by Lievense & Lim, 1982), and as a result respiring cells possess many well-developed mitochondria (Mian *et al.*, 1973; Ferdouse *et al.*, 1972; Fiechter *et al.*, 1972; cited by Lievense & Lim, 1982). TCA cycle intermediates are drawn off for biosynthesis, although there are reactions that replace them (anaplerotic reactions): the carboxylation of pyruvate occurs to provide oxaloacetate (Miller & Atkinson, 1972; Cazzulo, Claisse, & Stoppani, 1968; cited by Lievense & Lims, 1982); amino acids can be used to supply 2-oxoglutarate; lipids to provide acetyl-CoA; and the glyoxylate bypass serves in a limited capacity, to provide succinate and malate from acetyl-CoA (Beck & von Meyenberg, 1968; cited by Lievense & Lims, 1982) that is derived from a variety of substrates including ethanol.

Recent reviews covering intermediary metabolism include Entian and Barnett (1992); Gancedo and Serrano (1989); Young (1987); and Kappeli (1986).

1.2.3. Environmental Influences on Yeast Physiology

During a cell's interaction with its environment, the substrates, products, solutes, gases and other nutrient factors within the medium will influence the physiological state of the cell (Rose, 1987) that is itself limited by the genetic capability of the species. A schematic diagram of this concept is presented in Figure 1.1 below.

Figure 1.1: Schematic representation of the factors influencing the physiological state of the yeast cell.



Hence, cell behaviour and regulatory responses must be considered in relation to the environmental variables, and whilst due consideration should be given to the genetic basis of response, this study is primarily directed at the influence of environmental factors on the intermediary metabolism of yeast cells. Although this study is specifically concerned with the influence of magnesium availability on fermentative metabolism, a review of the functions and influences of other widely studied environmental factors, such as glucose concentration and oxygen availability (Rose, 1987; Kappeli, 1986), are necessary to place the role of magnesium in context.

Information on the control of metabolic pathways in *S. cerevisiae* is primarily arrived at by studying the effects of the glucose concentration and oxygen availability on the growth and enzyme levels of cells cultivated under precisely defined conditions (Young, 1987; Fiechter *et al.*, 1981). This connection between metabolism and culture conditions is highlighted by observations of the differences between batch and continuous culture data. In batch mode, where the glucose concentration is initially high, cells exhibit predominantly fermentative metabolism (von Meyenberg & Fiechter,

1966; cited by Lievense & Lim, 1982) whereas in continuous mode, under glucose limitation, respiratory metabolism predominates at low dilution rates, with a shift to fermentation occurring at higher dilution rates (Knopfel, 1972; von Meyenberg, 1969; cited by Lievense & Lim, 1982). Thus, culture conditions directly influence the cell's utilization of glucose with differences observed in the carbon-flow between modes of metabolism being governed by the oxygen status and the glucose concentration (Schatzmann, 1975; cited by Fiechter *et al.*, 1981). Much remains to be understood about the physiological requirements of *S. cerevisiae* (Quain, 1988), but clearly, inadequate culture conditions will lead to inefficient growth and inferior product formation, whilst the tailoring of the environment to the specific physiology of the yeast will ensure optimum performance and product formation, thus making media design of fundamental importance in biotechnological processes (Meyer, Kappeli, & Fiechter, 1985; Rose, 1987).

1.2.3.1. The Influence of Glucose on Yeast Physiology

Free glucose is considered a primary regulating element for yeast in the presence of oxygen (Fiechter *et al.*, 1981), and consequently its effects on microbial enzymes and its role as a regulatory compound has been widely studied (Gancedo & Serrano, 1989; Fiechter *et al.*, 1981). All known yeast species are capable of using glucose as a single source of carbon and energy, although this ability is oxygen-dependent (Barnett, 1976; cited by Kappeli, 1986); species are therefore grouped accordingly (Kappeli, 1986) as in Table 1.2 below.

Several regulatory phenomena pertaining to glucose have been discussed in the literature (e.g. Gancedo & Serrano, 1989; Fiechter *et al.*, 1981) and a brief review is presented here.

Table 1.2: Classification of yeasts according to their metabolic response to glucose and oxygen.

Classification	Glucose	Oxygen	Examples
Respirative yeasts; i.e. exhibit no fermentative activity.	Insensitive to free glucose levels.	Do not grow in the absence of oxygen.	*Trichosporon cutaneum* (Kappeli, 1986).
Obligately-fermentative yeasts; i.e. exhibit no respiratory activity.	Insensitive to free glucose levels.	Do not grow in the presence of oxygen.	*Candida tropicalis* (Fiechter *et al.*, 1981).
Facultatively-fermentative yeasts (Crabtree positive); i.e. ferment aerobically if excess glucose is present.	Sensitive to the free glucose levels.	Insensitive to free oxygen levels when excess glucose is present.	*S. cerevisiae* (Fiechter *et al.*, 1981).
Facultatively-fermentative yeasts (Crabtree negative); i.e. exhibits fermentative activity only in aerobic conditions.	Sensitive to the free glucose levels.	Sensitive to free oxygen levels.	*Hansenula nonfermentans* (Kappeli, 1986).

The Crabtree Effect

This regulatory phenomenon, originally described by Crabtree (1928), is believed to be exhibited by cells grown in batch cultures at high glucose concentrations or in glucose-limited continuous cultures at high dilution rates, which equate to high glucose concentrations, and is defined as a repression of respiratory activity by glucose under aerobic conditions, resulting in a deregulation of glycolysis and subsequent fermentative activity (Fiechter *et al.*, 1981). Threshold glucose concentrations for the operation of the Crabtree effect are greater than 0.005-0.01%w/v according to some authors (Woehrer & Roehr, 1981; Mian *et al.*, 1973; Knopfel, 1972; cited by Lievense

& Lim, 1982) or greater than 0.01-0.02%w/v according to van Dijken *et al.* (1990). Higher thresholds still were originally reportedly, 0.02-0.1%w/v (Richard & Hoggan, 1978; Suomalainen, *et al.*, 1973; Hauklie & Lie, 1971; Prescott & Dunn, 1949; cited by Jones, Pamment, & Greenfield, 1981), whilst Young (1987) reports cessation of respiration in *Saccharomyces* strains at a free glucose concentration of less than 0.4%w/v.

Swanson and Clifton (1948; cited by De Deken, 1966) originally argued that in batch cultures, *S. cerevisiae* could use fermentation as a mechanism for growth during aerobiosis whilst Lemoigne, Aubert, and Millet (1954; cited by De Deken, 1966) linked the glucose concentration to the presence or absence of a diauxic response. These findings were expanded by Slonimski (1954; cited by De Deken, 1966) who showed that 'respiratory adaptation' of cells occurred as a function of the glucose concentration; i.e. respiration reached a peak at 6×10^{-3}M glucose, whereupon the pathway's activity declined and fermentation started to take precedence. This data was supported by the observation that the Crabtree effect was operational at the start of the exponential phase of growth, when glucose concentrations were relatively high, but its influence was reduced towards the end of the phase when glucose concentrations had fallen (Ephrussi, Slonimski, Yotsuyanagi, & Tavlitzki, 1956; cited by De Deken, 1966).

De Deken (1966) proposed that the predominance of one mode of metabolism occurred at the expense of the other, and that the cause was a repression of the activities and synthesis of the respiratory enzymes. Hence, the effect became considered to be a form of catabolite repression upon the oxidative pathways of the cell precipitated by high glucose levels, irrespective of the presence or absence of oxygen (Fiechter *et al.*, 1981; Crabtree, 1928). The repression has a widespread impact including changes in the composition, levels and activity of the chief enzymes of the TCA cycle, the electron-transport chain, the glyoxylate bypass, and the gluconeogenic pathway (McDonald & Tsai, 1989; Young, 1987). Concerning the enzymes of the oxidative pathways, the change from de-repression to repression of enzyme synthesis, can result

in a 5- to 200-fold decrease in their resultant activities depending on the specific enzyme (Fiechter *et al.*, 1981).

However, it now appears as though catabolite repression is only one of several regulatory mechanisms available to the cell that can include alterations in protein synthesis, modification of enzymes and changes in enzyme turnover rates. The latter mechanism, the degradation of proteins by proteolytic enzymes as part of enzyme-turnover, is known as catabolite inactivation and was proposed after the observation that the kinetics of repression of the respiratory pathways exist in high as well as low time-constants (Holzer, 1976; cited by Young, 1987). The finding that an isoenzyme of malate dehydrogenase from *S. cerevisiae* is inactivated by a proteolytic process (Hagele, Neff, & Mecke, 1978; cited by Young, 1987) further supported the idea of inactivation, as did the recognition of several proteolytic enzymes within yeast vacuoles (Meussdoefer, 1978; cited by Fiechter *et al.*, 1981) which are subject to glucose repression (Hansen, Switzer, Hinze, & Holzer, 1977; Frey & Rohm, 1977; cited by Fiechter *et al.*, 1981). Takeda (1981) provided further evidence, based on inhibitor studies, that supported the idea of glucose-induced, selective proteolysis as a control mechanism with especial regard to the inactivation of mitochondrial enzymes. Investigations focus on the control of the proteinases themselves, including their inactivation (Fiechter *et al.*, 1981) and on the targeting of enzymes to be inactivated by phosphorylation (Gancedo & Serrano, 1989). Wolf (1986) provides a concise review of the involvement of proteinases in cellular control.

Although the precise transducers of the Crabtree effect are unknown, glucose is not seen as the primary effector since it does not accumulate to any extent inside the cell (Suomalainen & Oura, 1971; Kotyk & Janacek, 1970; cited by Lievense & Lim, 1982). Other metabolites that have been considered include ATP and NADH (von Meyenberg, 1969; Polakis *et al.*, 1965; cited by Lievense & Lim, 1982); TCA cycle intermediates (Mor & Fiechter, 1969; cited by Lievense & Lim, 1982); glucose-6-phosphate (Gorts, 1971; cited by Lievense & Lim, 1982); and cyclic AMP (Fiechter *et al.*, 1981). Further, the addition of nucleotides, cAMP included, can reverse the

Crabtree effect in repressed cells (Fang & Butow, 1970; cited by Lievense & Lim, 1982). Comparisons to prokaryotic models of cAMP regulation have been made but due to the lack of data available for yeast cAMP systems (Eraso & Gancedo, 1984; Matsumoto, Uno, Ishikawa, Oshima, 1983), reservations have been maintained (Fiechter *et al.*, 1981) and are proving well-founded (Gancedo & Serrano, 1989). The regulatory domain of a hexokinase isozyme, HK PII, has been implicated in the expression of the glucose repression (Niederacher & Entian, 1987; Entian & Frohlich, 1984).

Barford and Hall (1981; cited by Lievense & Lim, 1982) have reported results in glucose-limited continuous culture that dispute some aspects of the Crabtree effect. Their cultures showed an adaptive saturation of oxidative metabolism when held at a fermentative dilution rate, with no actual repression of respiration being observed, although repression was seen during the exponential phase of a batch culture. Previously, Karrer (1978; cited by Fiechter *et al.*, 1981) had found that the critical dilution rate at which glucose repression sets in is strongly dependent on the glucose concentration of the incoming media in the continuous culture. This is not only in direct contradiction to the theoretical expectations of continuous culture, but also invites a reconsideration of Schatzmann's (1975) argument that a definable glycolytic flux exists, corresponding to a specific dilution rate, at which repression sets in. The argument that regulation is dynamic in nature and that repression is dependent on metabolic rates rather than glucose concentration alone (Beck & von Meyenberg, 1968; cited by Lievense & Lims, 1982) is also pertinent. Further, Brandli (1980; cited by Fiechter *et al.*, 1981) observed a shift in the onset of repression due to an Fe-Cu limitation in a medium used for studies of *Schizosaccharomyces pombe*; i.e. the limitation induces repressed growth at low dilution rates where the glycolytic flux is much less than the flux observed at the onset of repression in the non-limited medium. The conclusion is that the onset of repression can be altered by changes in the growth-rate limitations existing within the environment. Fiechter *et al.* (1981) argued that

significant physiological changes occur in response to a limited capacity of cellular metabolism that is governed and introduced by these environmental limitations.

Rieger *et al.* (1983) observed in continuous culture that an upper limit to the oxygen uptake rate was reached during growth on glucose and on glucose plus ethanol that, together with the fact that ethanol consumption appeared to be limited by this respiration rate, led the authors to hypothesize a "limited respiratory capacity" within the cells. The pulsing of excess glucose into a continuous culture of respiring *S. uvarum* cells caused no repression of respiration but did induce a fermentative response (Petrik *et al.*, 1983) and it was concluded that an overflow reaction was taking place at pyruvate, a hypothesis in keeping with the kinetic data of the two enzymes as detailed in section 1.2 above. McDonald and Tsai, (1989) provide a succinct summary of the above argument.

An upwards shift in dilution rate from a respirative range to a more fermentative range revealed a long-term adaptation of cellular physiology (Petrik *et al.*, 1983) with a decrease in the mitochondrial cytochrome content. The overflow reaction, known as 'the short-term Crabtree effect' and the subsequent physiological adaptation is believed to be caused by two separate mechanisms (Kappeli, 1986).

Barford (1985) similarly proposed that there is a maximum respiratory capacity existing within the cells, whilst Kappeli (1986) summarizes the types of glucose metabolism distinguishable in yeast cells according to the prevailing environmental conditions, and proposes that the limited respiratory capacity is the basis for 'respiro-fermentative growth'. During respiro-fermentation, saturation of the respiratory pathway results in an overflow of pyruvate towards the fermentative pathway which in turn causes changes in the cellular concentration and/or production rate of metabolites such as NADH (Beyeler, Einsele, & Fiechter, 1981; cited by Kappeli, 1986), carbon dioxide (Kappeli, Arreguin, & Rieger, 1985) and ethanol that may serve as effectors for the long-term physiological changes. Kappeli (1986) notes that the idea of repression as a mechanism for the regulation of glucose metabolism emerged before the recognition (Petrik *et al.*, 1983) of the above-mentioned physiological states, and argues

that whilst repression may be a regulatory option for the certain enzymes (for examples, see Gancedo & Serrano, 1989) it is insufficient in explaining the non-repressed physiological states observed in continuous culture.

The conclusion offered by Kappeli (1986), is that glucose utilization in *S. cerevisiae* and related yeasts proceeds by several pathways whose relative contribution is dependent on the prevailing environmental conditions and their influence on firstly the respirative capacity of the cell and secondly the magnitude of the fermentative pathway, and that respiro-fermentative metabolism can account for phenomena that have hitherto been explored as regulatory devices in their own right. The Crabtree effect is seen as being the initial attempt to explain respiro-fermentative metabolism by way of repression mechanisms and the "Pasteur Effect" is viewed as being a premature triggering of respiro-fermentative behaviour by oxygen limitation. The latter view indicates that the cell's respiratory capacity can be decreased by the reduction of oxygen levels (and eventually extinguished under anaerobic conditions) which is in accord with Karrer (1978) and Brandli's (1980) findings (cited by Fiechter *et al.*, 1981), mentioned above. Kappeli (1986) suggests that glucose repression may be a mechanism for the long-term adaptation of certain enzymes during respiro-fermentative metabolism that yields a given physiological state, and that the allosteric control mechanism of phosphofructokinase (section 1.2.3.2: The Pasteur Effect) will still be of fundamental importance in glycolysis. A schematic representation of the possible co-operative behaviour of these mechanisms has been presented by Kappeli (1986).

Comparative studies of a Crabtree-positive yeast (*S. cerevisiae*) and a Crabtree-negative yeast (*C. utilis*) during glucose pulsing into a glucose-limited chemostat showed that the pyruvate dehydrogenase activities and the kinetics of mitochondrial pyruvate oxidation are similar (van Urk *et al.*, 1989a and 1989b) but differences between the levels of pyruvate decarboxylase and the sensitivity of the enzyme to inorganic phosphate exist (van Urk *et al.*, 1989b). It is suggested (van Urk *et al.*, 1989b) that the increased glycolytic activity after the pulse gives increased levels of pyruvate that are used by the higher levels of pyruvate decarboxylase present within the

cells of *S. cerevisiae*. The decrease in phosphate levels commensurate with the increased glycolytic activity releases the pyruvate decarboxylase of *S. cerevisiae* from allosteric inhibition to a greater extent than would occur within *C. utilis* further enhancing the contribution of the fermentative pathway within *S. cerevisiae* (van Urk *et al.*, 1989b).

The Custer Effect

Scheffers (1966) coined this phrase to describe a negative Pasteur effect observed in many glucose-fermenting yeasts (Scheffers & Wiken, 1969) whereby the fermentation of glucose is inhibited during anaerobiosis (Custers, 1940; Wiken, Scheffers, & Verhaar, 1961; cited by Scheffers, 1966). Whilst its extent appears to be influenced by the initial glucose concentration (Carrascosa, Viguera, Nunez de Castro, & Scheffers, 1981), trace quantities of oxygen or organic H-acceptors remove the effect (Scheffers, 1966). Scheffers (1966) postulated that the effect may be due to a shortage of NAD, whilst Scheffers (1979) showed that, under anaerobic conditions, the ratio of NAD-to-NADH falls sharply upon the addition of glucose to the culture. Scheffers and Misset (1974) hypothesized that the production of acetic acid and the concomitant reduction of NAD may be the mechanism for the effect, whilst Carrascosa *et al.* (1981) further implicated the activity of the NAD-linked aldehyde dehydrogenase by showing that, in *Brettanomyces* species, the acetate is not transformed to acetyl-CoA but is instead largely excreted into the medium, which is not the case in *Saccharomyces* species. The mechanism behind this behaviour is not clearly understood but it appears that reduced activity of the acetyl-CoA synthetase is not responsible (Carrascosa *et al.*, 1981). Wijsman *et al.* (1984) provided further evidence that the Custer effect is due to an imbalance of redox couplers as a result of acetic acid production and the organism's inability to produce glycerol.

Scheffers (1979) states that *S. cerevisiae* exhibits a transient Custer effect when glucose is introduced to an anaerobic culture of cells grown on ethanol but does not exhibit an effect under aerobic conditions growing on glucose.

Glucose Transport

The transport of glucose across the cell membrane is seen as a likely regulation point for glycolysis (Serrano & DelaFuente, 1974; Kotyk & Kleinzeller, 1967), as glucose does not accumulate to any extent within the cell thus indicating a sufficiency of metabolism over transport (Becker & Betz, 1972; cited by Gancedo & Serrano, 1989). There are two transport systems described (Gancedo & Serrano, 1989), the first with a low K_m (1mM) and the second with a high K_m (20mM), with their presence being dependent on the hexose kinases present in the cell (Bisson & Fraenkel, 1983; cited by Gancedo & Serrano, 1989). Glucose transport has been implicated in the Pasteur effect with the suggestion (Salas, Vinuela, Salas, & Sols, 1965; cited by Lagunas & Gancedo, 1983) that the accumulation of glucose-6-phosphate during the inhibition of phosphofructokinase results in the observed (Serrano & DelaFuente, 1974) decrease in the affinity of the carrier for glucose (Alonso, Pascual, Romay, Herrara, & Kotyk, 1989) under aerobiosis, although this concept has been questioned by Lagunas and Gancedo (1983).

1.2.3.2. The Influence of Oxygen on Yeast Physiology

The involvement of oxygen in brewery fermentations has long been established (Brown, 1914; cited by Tyagi, 1984), with studies revealing its influence on many aspects of cell metabolism including viability, reproductive ability, growth and the production of flavour metabolites such as acetate, pyruvate, esters and higher alcohols (Kirsop, 1974). As Jones *et al.* (1981) point out, molecular oxygen in *S. cerevisiae* has several aspects to its involvement (Fiechter *et al.*, 1981) both in biosynthetic and catabolic pathways, which complicates the understanding of its influence on the cell and the distinction between any regulatory functions and its role as a nutrient component.

The role of oxygen in ethanol fermentation is not well understood (Tyagi, 1984), and whilst traditionally fermentation is an anaerobic process, it is known that trace quantities of oxygen can stimulate fermentation rates (Wiken, 1961; White & Munns, 1951; cited by Tyagi, 1984), the kinetics of growth and the final yield (Pironti, 1971;

cited by Tyagi, 1984) through its involvement in the synthesis of membrane components (Kirsop, 1974). Structurally-significant unsaturated fatty-acyl residues in lipids, sterols (Andreasen & Stier, 1954; cited by Quain, 1988) and nicotinic acid (Tyorinoja, Nurminen, & Suomalainen, 1974; cited by Rose, 1987) require molecular oxygen for their synthesis by the yeast both in the starter culture and at the beginning of the main fermentation (Thurston, Quain, & Tubb, 1982; cited by Quain, 1988). Availability of these compounds will influence subsequent growth and performance (Quain, 1988; Jones *et al.*, 1981) according to strain type (Kirsop, 1974). Hunter and Rose (1971), and Tyagi (1984) have reviewed the interaction of growth and cell metabolism with fatty acids and sterols during wort fermentations. Yeast propagation in the presence of oxygen can further affect the structural composition of the yeast, whereby the oxygen changes the morphology of the cells by affecting the nitrogen utilization of the yeast (Jones & Pierce, 1964; cited by Tyagi, 1984) which alters the carbon-to-nitrogen ratio (Kirsop, 1974), a factor shown to alter cell shape (Brown & Hough, 1965; cited by Kirsop, 1974).

Yeast respiratory activity has a high requirement for disposable molecular oxygen as a final electron acceptor within the respiratory chain as reducing power from the TCA cycle is passed to nascent ATP. Originally, it was thought that oxygen's role was passive, acting as a catalyst that activated the hydrogen of the oxidation reactions (Horecker, 1978), but when Warburg (1949; cited by Horecker, 1978) explored yeast respiration and the involvement of oxygen and iron using cyanide as an inhibitor, differences were observed between the respiration rates of brewer's yeast and wild yeasts, and the conclusion was made that the rate of respiration depended on the oxygen-conditions of the culture (Warburg, 1949; cited by Horecker, 1978). Nevertheless, it has been argued that the presence of oxygen becomes energetically irrelevant to *S. cerevisiae* growing on glucose (growth on ethanol is possible only if oxygen is present), as 95% of the substrate is fermented (Lagunas, 1979) under high glucose concentrations due to the Crabtree effect (Lagunas, 1982).

The Pasteur Effect

Pasteur originally made the observation that yeasts under aerobic conditions consumed significantly less sugar to form the same quantity of yeast as was formed under anaerobic conditions (Pasteur, 1861) concluding that fermentation is inhibited in the presence of oxygen (Pasteur, 1867; cited by Fiechter *et al.*, 1981). After first being observed in yeasts, the phenomenon has subsequently been recorded in skeletal and heart muscle, brain and liver tissue, the kidney cortex, Novikoff hepatoma and adenocarcinoma (Tejwani & Ramaiah, 1971; cited by Tejwani, 1978).

The phenomenon was termed by Warburg (1926; cited by Racker, 1974) as the Pasteur Effect and subsequently became interpreted as meaning a decrease in the glucose uptake rate during respiration (Burk, 1939; cited by Racker, 1974), shown experimentally to be the case in 'resting' cells (Lynen, Hartmann, Netter, & Schuegraf, 1959; cited by Racker, 1974) but not in growing cells (Schatzmann, 1975; cited by Fiechter *et al.*, 1981). The Pasteur effect was recognised as indicating the presence of a regulatory system on the EMP pathway (Gancedo & Serrano, 1989), although in reviewing the history of the effect, Racker (1974) noted that there was much confusion and controversy regarding its role as a control mechanism, and that measurements of end-products rather than studies on glucose-uptake originally distracted thinking from the areas at which control might operate. This view was also maintained by Fiechter *et al.* (1981) who argued that the cell type and their uptake mechanisms, as well as the experimental conditions, influenced the differences observed between resting and growing cells.

The Pasteur phenomenon is now described as an inhibition of the glycolytic pathway in the presence of oxygen resulting in an inhibition of fermentation (Fiechter *et al.*, 1981), which is to be seen as a control mechanism, operating at several levels, that balances the competition between fermentation and respiration for substrate (Racker, 1974). The idea was originally held (Lynen, 1941; Johnson, 1941; cited by Fiechter *et al.*, 1981) that this competition by the two modes of metabolism is for the available inorganic phosphate, which becomes limiting during oxidative phosphorylation as ATP

is synthesized. However, this view was regarded as insufficient although essentially correct and other mechanisms were explored (Fiechter *et al.*, 1981).

The role of phosphofructokinase was first hypothesized by Engelhardt and Sakov (1943; cited by Tejwani, 1978) because of its known activation under anaerobic conditions and its effect on the activities of hexokinase and pyruvate kinase, whilst further work implicated it more fully as a mechanism of the Pasteur effect (Sols, 1968; cited by Fiechter *et al.*, 1981) through its allosteric properties (Sols, 1981; cited by Gancedo & Serrano, 1989). For example, the inhibition or activation of phosphofructokinase by ATP and AMP respectively (Sols, Gancedo, & DelaFuente, 1971; cited by Young, 1987) alters the glucose flux through the pathway during respiration, as does the depletion of intracellular inorganic phosphate during respiratory ATP synthesis (Lynen, 1963; cited by Young, 1987). There are 20 metabolites currently known to regulate the activity of phosphofructokinase. For example, the inhibitors ATP, citrate and Mg (Vinuela, Salas, & Sols, 1963; cited by Gancedo & Serrano, 1989; Salas *et al.*, 1965; Sols, 1976 respectively; cited by Lagunas & Gancedo, 1983) and the promoters AMP, ammonium and phosphate (Ramaiah, 1974; Sols & Salas, 1966; Banuelos, Gancedo, & Gancedo, 1977 respectively; cited by Lagunas & Gancedo, 1983), many of which are synergetic in nature (Tejwani, 1978) but which require the quantification of their relative contributions (Racker, 1974). Work in this area has been carried out (Lagunas & Gancedo, 1983) which has questioned the regulatory role of some proposed effectors such as fructose 2,6-bisphosphate, AMP and citrate, whilst intracellular compartmentation effects remain an issue (Lagunas & Gancedo, 1983; Racker, 1974).

It is now generally accepted that the integrated multiple-allosteric controls of phosphofructokinase represent a key mechanism in regulating the glycolytic flux (Fiechter *et al.*, 1981) by way of the feedback inhibition on hexokinase by fructose 6-phosphate. This is accepted to be the situation for many cell types in a variety of physiological states (Fiechter *et al.*, 1981) despite conclusions to the opposite (Racker, 1976; cited by Fiechter *et al.*, 1981). For example, the presence of a second,

constitutive form of phosphofructokinase found in yeasts (Gancedo & Serrano, 1989) which is not susceptible to ATP inhibition may reduce the EMP's susceptibility to the influence of oxygen whilst in animal cells the inhibition of hexokinase by glucose 6-phosphate is considered a complementary regulatory mechanism (Crane & Sols, 1954; cited by Lagunas & Gancedo, 1983), although yeast hexokinase is not similarly effected. Other evidence for the involvement of phosphofructokinase in the regulation of glycolysis is reviewed by Gancedo and Serrano (1989).

The differences between growing cells and resting cells are now seen as being a response of the former to all the complex control loops implied by an active respiratory chain. Also, growing cells are prone to the respiratory repression imposed by glucose whereby ATP generation is dramatically cut and subsequent release from allosteric control is substantial, thus over-shadowing any glycolytic regulation. Galactose-grown cells, where respiratory repression is minimized because of the inefficient galactose transport system, reveal a Pasteur effect of 50% of the level seen in resting cells, an observation that indicates other, unknown regulatory mechanisms operating in the resting cells (Fiechter *et al.*, 1981).

The Kluyver Effect

This effect was named by Sims & Barnett (1978) after a report by Kluyver & Custers (1940) that certain yeast species can utilize particular glycosides aerobically but not anaerobically despite being able to ferment the component hexoses anaerobically (Sims & Barnett, 1978). Observed in many yeast species such as *Candida* and *Kluyveromyces* but not seen in *Saccharomyces* (Sims & Barnett, 1978), it was suggested that the effect was the result of the direct or indirect requirement for oxygen of the particular glycosidic transport mechanism (Sims & Barnett, 1978). Species-specific responses are observed (Sims & Barnett, 1978), as for example in *C. utilis* which cannot ferment xylose anaerobically despite being able to do so aerobically (Bruinenberg *et al.*, 1983). The transport system was not considered to be responsible but rather the effect

was believed to be as a result of a redox imbalance of the cofactor, NADH (Bruinenberg *et al.*, 1983).

1.2.3.3. The Influence of Ethanol on Yeast Physiology

Ethanol, whilst obviously being the main reason for conducting a fermentation, is often considered only in its role as a toxic compound rather than as a medium nutrient.

Many studies attribute ethanol as a prime cause of the reduction in fermentative activity (Ingram & Buttke, 1984; Moulin *et al.*, 1984; Maiorella, Blanch, & Wilke, 1983; Millar, Griffiths-Smith, Algar, & Scopes, 1982), with both nutritional limitations for unsaturated membrane lipids (Casey & Ingledew, 1986) and magnesium (Dombek & Ingram, 1986b) exacerbating the decline (Dombek & Ingram, 1986b; Casey, Magnus, & Ingledew, 1984). Removal of accumulated ethanol does not restore fermentative activity, whilst exogenously-added ethanol does not affect the fermentative abilities of the cells to the same extent as endogenously-produced ethanol (Dombek & Ingram, 1986a).

Other investigations into the changes occurring during the transition from exponential to stationary phase centre on the action of allosteric effectors such as AMP, a competitive inhibitor of hexokinase relative to ATP, and show how hexokinase activity and the rate of ethanol production decline in parallel as a function of ATP/AMP concentrations (Dombek & Ingram, 1988). This work was extended to include other key glycolytic enzymes (Alterthum, Dombek, & Ingram, 1989).

Tolerance to ethanol is strain dependent, with growth being retarded above 10%w/v, whilst ethanol production is affected above 20%w/v (Ismail & Ali, 1971; Ranganathan & Bhat, 1958; Troyer, 1953; Gray, 1941; cited by Jones *et al.*, 1981). Toxicity is most strongly observed on the cell membrane (White, 1978; cited by Jones *et al.*, 1981) although its effects are seen in other cell physiological functions (Ismail & Ali, 1971; Ismail & Hegazy, 1970; cited by Jones *et al.*, 1981) such as enzyme activity, chiefly alcohol dehydrogenase (Brown, 1976; cited by Jones *et al.*, 1981) and hexokinase (Nagodawithana *et al.*, 1977; Gray & Sova, 1956; cited by Jones *et al.*,

1981). The level of toxicity experienced by the cell is dependent on other environmental considerations (Jones *et al.*, 1981) such as sugar concentration and temperature (White, 1978; Nagodawithana & Steinkraus, 1976; Chye & Meng, 1975; Kunikee & Amerine, 1968; Gray,1941; cited by Jones *et al.*, 1981).

1.2.3.4. The Influence of Other Environmental Factors on Yeast Physiology

pH affects yeast cell growth and metabolite production (Buzas, Dallmann, & Szajani, 1989) with a fermentation optimum around pH 4 (Buzas *et al.*, 1989) possibly due to the effect on plasma membrane-bound proteins (Rose, 1987). pH also influences the kinetics of cell transport mechanisms (Jones & Gadd, 1990).

Weak acids, such as acetic and propionic acid, can in the absence of oxygen cause a stimulation of fermentation, as the cell requirement for ATP to pump out the excess protons increases. In the presence of oxygen, the cells obtain the required ATP by enhancing respiration; however, at a critical concentration of acid, fermentation begins to replace respiration (van Dijken *et al.*, 1990), whilst an excess of the acid causes intracellular acidification which in turn initiates a general proteolysis (Lampen, 1966; cited by Gancedo & Serrano, 1989). Rose (1987) has provided further information on the growth-inhibiting action of organic acids. The amount of ATP required to counteract the acid's effect is the same for aerobic or anaerobic conditions (van Dijken *et al.*, 1990).

The relative concentration of acetyl-CoA and CoASH, known as the "acetyl charge", may be important in regulating intermediary metabolism (Quain, 1988).

Biological compartmentation as a way of effecting changes in intermediary metabolism has the proton gradient of Mitchell's chemi-osmotic theory as a prime example, whilst the separation of the component parts of intermediary metabolism throughout the mitochondria is thought to be an extra level of control open to the cell (Jones & Gadd, 1990).

NAD(H) is involved in catabolic processes, whereas NADP(H) functions mainly in anabolic processes as produced by the pentose phosphate pathway and it is proposed that the regulation of their respective redox levels could serve an important function within the physiology of the cell (Bruinenberg, 1986).

The reaction of yeasts to other chemicals present within the environment, such as nitrogen, phosphorous and sulphur, are covered by Rose (1987).

1.2.3.5. Metal Ions in Yeast Physiology

Inorganic ions are required in micromolar and millimolar concentrations for the growth and metabolism of micro-organisms (Hughes & Poole, 1989) and any imbalance in ionic availability is reflected in complex and subtle changes of the cell's metabolism (Casey & Ingledew, 1986; Jones & Greenfield, 1984). General microbial inorganic nutrition has been reviewed (Hughes & Poole, 1989; Hutner, 1972) as have yeast inorganic requirements (Jones & Gadd, 1990; Casey & Ingledew, 1986; Jones & Greenfield, 1984). The following is a brief survey of general microbial nutrition, the term 'yeast' referring to *Saccharomyces* species except where stated as otherwise.

Potassium and magnesium cations (K^+ and Mg^{2+} respectively) are considered as bulk intracellular species whilst sodium (Na^+) and calcium (Ca^{2+}) together with zinc (Zn^{2+}) and the 3*d* transition metals, such as copper (Cu^{2+}), manganese (Mn^{2+}), cobalt (Co^{2+}) and iron (Fe^{2+}) are considered as being present only at trace levels. These arbitrary designations are based on a combination of the concentration at which microbial growth is limited and the total levels found within the cell. The amounts required for growth are not necessarily identical to the amounts needed for metabolic processes such as secondary metabolite production whilst Jones and Greenfield (1984) state that for yeast, the trace elements show only a minor effect on cell yield but a more significant effect on cell viability and fermentation rate (Pis, 1969; Pis, 1962; cited by Jones and Greenfield, 1984).

Cationic effects within the cell cover a range of functions that include: stabilizing anionic charges within biological structures; cross-linking bio-polymers and influencing

their orientation; maintaining the osmotic balance; providing charge and concentration gradients; catalysing enzymic reactions and providing a mechanism for redox exchange (Hughes & Poole, 1989, Williams, 1976). The bulk cations tend to be involved in establishing the required cationic environment of the cell and its enzyme systems and maintaining the osmotic balance with their uptake being strongly influenced by the organism's growth rate whilst the trace cations, usually transition metals, tend to have more specific biological functions in metabolism and/or within cellular structures (Jones & Greenfield, 1984).

Functions of Metal Ions

The biological action and function of any cation will reflect its intrinsic chemical properties (Hughes & Poole, 1989; Williams, 1970; Steinberg, 1938) including ionic charge and ionic radius (i.e. charge density); electronic configuration, electron affinity and ionic structure (i.e. redox properties); the rates of ligand exchange and degree of hydration (i.e. binding ability). For example, the mid-transition metals such as copper, iron, cobalt and molybdenum will act as static redox catalysts; the end-transition metal zinc will function as a static super-acid catalyst and the alkali and alkaline metals will be involved in structural roles and the transport of ionic-charge (Williams, 1976). These fundamental chemical properties can be used to understand biological function in areas such as ion-protein binding (Williams, 1970), loss of protein activity during fractionation processes (Hutner, 1972) or even media design where the improper specification of salts may lead to irreproducibility due to precipitate formation (Hutner, 1972).

The nature of the ligand will also influence the biological functioning of the cation. For example, calcium exhibits a preference for oxygen-containing groups whilst magnesium shows a very slight preference for nitrogen-bearing species that ensures their association with different membrane components (Lewis, Sommer, & Patel, 1978). Williams (1979) has reviewed cationic interactions with proteins and membranes, whilst Hughes and Poole (1989) have reviewed the chemical groups

capable of functioning as cationic ligands within the three major classes of macromolecules: proteins, nucleic acids and polysaccharides.

Reviews of specific microbial requirements and uses of individual metals are available: cations (Jones & Gadd, 1990; Hughes & Poole, 1989; Jones & Greenfield, 1984; Pirt, 1975; Hutner, 1972); manganese (Silver & Jasper, 1977; cited by Hughes & Poole, 1989); zinc (Sigel, 1983; Failla, 1977; cited by Hughes & Poole, 1989); and vanadium (Ramasarma & Crane, 1981; cited by Hughes & Poole, 1989).

Jones and Greenfield (1984) note that the effects of cations on the yeast cell are probably poorly characterized because of their multiple roles within the cell, i.e. structural and enzymic functions both within the cytosol and the plasma membrane. Jones and Gadd (1990) reiterate this view and include a review of intracellular compartmentation and the possible physiological mechanisms involved in internal ion transport as well as the feasibility of these mechanisms providing an extra level of metabolic control to the cell (Jones & Gadd, 1990).

Cations are structurally important for shielding or neutralising the anionic charges of proteins and nucleic acids, e.g. potassium and magnesium (Tempest, 1969; cited by Jones & Greenfield, 1984), whilst membrane phospholipids (Lewis *et al.*, 1978; Romkes & Lewis, 1971; Indge, 1968) use calcium and magnesium as the primary effectors (Armstrong, 1972; Meryman, 1972; cited by Jones & Greenfield, 1984) although zinc is believed to be critically involved (Bettger & O'Dell, 1981; cited by Jones & Greenfield, 1984).

The absolute concentrations of potassium and magnesium as well as their relative ratio have essential functions in the stabilization of nucleic acids and ribosomes and in the activation of key enzymes involved in the synthesis of the nucleic acids although potassium's main role is in the control of the osmotic balance of the cell as a charge-compensator for small anions. This interaction with small anions such as the phosphate groups located on the surface of nucleic acids is reviewed by Eichhorn and Marzilli (1981; cited by Hughes & Poole, 1989).

Sodium gradients established across microbial cell membranes by energy-dependent sodium pumps or Na^+/H^+ antiports are used to transport nutrients into the cell but no positive metabolic role for sodium during yeast growth or fermentation has been reported (Jones & Greenfield, 1984) with yeasts using proton gradients to transport monovalent cations, cationic amino acids, calcium and magnesium across the plasma membrane (Pena, Gutierrez, Amo, & Schnabel, 1987).

Potassium, calcium and zinc ions have been shown to have structural significance within specific enzymes where they prevent the unfolding and denaturation of the protein (Matthews & Weaver, 1974; Matthews, Weaver, & Kester, 1974; cited by Hughes & Poole, 1989) or induce conformational changes necessary for the configuration of the active site (Bostian, Betts, Man, & Hughes, 1982; cited by Hughes & Poole, 1989) by the permanent cross-linking of chemical groups in a manner analogous to disulphide bridges (Hughes & Poole, 1989). Formation of temporary cross-links between enzyme and substrate can be used by the cell to obtain accurate relative orientations whilst the lability of the cross-linking of carboxylate groups present on the cell wall can be used to control cell permeability.

In yeasts, the majority of the calcium present within the cell is located in the cell wall and plasma membrane where it is involved in regulating lipid/protein interactions and maintaining structural integrity (Jones & Greenfield, 1984). Membranes have been shown to be protected from the disrupting effects of polyenes and butanol by calcium ions (Caraffoli, Balcavage, Lehninger, & Matoo, 1970). The structural role of zinc within yeast cells appears to be complementary to calcium in influencing membrane performance whilst the structural influence of potassium within the yeast cell is speculated as being limited to general charge-shielding (Jones & Greenfield, 1984).

Calcium ions are also specifically required during the flocculation of yeast cells (Stratford, 1989; Taylor & Orton, 1973 and 1975) although the actual mechanism is still a subject of enquiry (Stratford & Brundish, 1990).

Copper, cobalt, iron and manganese do not have a known structural role in yeasts.

Cations have a variety of functions within enzyme systems; as a catalytic centre; as an activator; as a stabilizer; as a redox catalyst or as an antagonistic 'mediator' between other activators and deactivators (Jones & Greenfield, 1984).

The discrimination of function will be due to the aforementioned chemical properties of each ion. For example, zinc is known to be a co-factor in over two hundred enzyme systems (Hughes & Poole, 1989; Densky, Gray, & Buday, 1966) possibly because of its intrinsic ability to adopt distorted co-ordination geometries which is essential for accommodating a succession of reaction intermediates. However, it is not redox reactive (Hughes & Poole, 1989) unlike iron and copper that are involved in the catalysis of electron-transfer and the oxidation/reduction of substrates. These redox reactions take place because of the ability of the transition metals to undergo rapid ligand-substitution and adopt alternative geometries and co-ordination numbers whilst other influencing factors include the range of oxidation states available and the flexibility of the redox potential of the metal-ligand complex according to the co-ordination environment (Hughes & Poole, 1989). The latter factor, the ability to tune the redox potential to a specific value is effected by a variety of mechanisms: the chemical properties of the ligands; electrostatic interactions on the redox centre by surrounding polar groups; and the presence of solvent(s) around the redox centre. Moore, Pettigrew, & Rogers (1986) provide a general review of this subject; as cied by Hughes & Poole (1990).

Numerous microbial enzymes are activated by potassium (Suelter, 1970; cited by Hughes & Poole, 1989) including many involved in protein synthesis and the catabolic processes, especially during yeast fermentation that appears to require twice the quantity required by respiring cells. Although little is known about the mechanisms' potassium uses to exert its catalytic effect it is thought that it may bind to enzymes in such a way as to stabilize the conformation (e.g. yeast aldehyde dehydrogenase (Wampler & Westhead, 1968; cited by Hughes & Poole, 1989)) or generate the active site (Lewis *et al.*, 1978; van Engel, 1969; Wyatt, 1964; cited by Jones & Greenfield,

1984) or, as has been suggested for pyruvate dehydrogenase, it may act as a bridge between enzyme and substrate to control the relative orientations.

Calcium is known to inhibit microbial ATPase (Caraffoli *et al.*, 1970). Whilst the requirement of *S. carlsbergenis* during growth in minimal media is believed to be for structural purposes only (Lotan, Berdicevsky, Merzbach, & Grossowicz, 1976), the significantly lower growth requirement of *S. cerevisiae* for this ion is not essential. Calcium is also reported to increase the fermentation efficiency of *S. bayanus* possibly by fortifying the cell membrane and reducing its susceptibility to the toxicity of ethanol (Nabais, Sa-Correla, Viegas, & Novais, 1988).

As stated, zinc is necessary for the activation of many yeast enzymes, (including GA3(P) dehydrogenase, ADH, aldolase, *etc.*), most of which are involved in metabolism, e.g. riboflavin synthesis and the activation of phosphatases. The zinc requirement is absolute, with any media deficiency incapacitating cell growth and fermentation resulting in the 'sticking' of brewery fermentations.

Copper is required for the activation of yeast enzymes including the phosphatases; a group of enzymes also activated by cobalt and zinc. The involvement of cobalt is not well understood but may be involved in cytosolic metabolism and membrane processes whilst the role of manganese appears to be metabolic, being required by such enzymes as yeast adenylate cyclase.

Iron is required in microbial redox reactions catalysed by the haeme-proteins (e.g. cytochromes) and non-haeme sulphur proteins. *Saccharomyces* species require a minimum of 0.3μM for the activity of the Fe^{3+}-dependent acid phosphatase (Arnold, Evans, & Denniston, 1983; cited by Hughes & Poole, 1989).

Transport of divalent cations and phosphate involves potassium as a counter-ion and therefore the intracellular concentration of this ion may be involved in transport regulation. Increased intracellular levels of potassium causes an increase in the yeast glycolytic flux and possibly controls the balance between fermentation and respiration by elevating the levels of ATP, ADP, phosphate and NADPH (Wumplemann &

Kjaergaard, 1979; Nordheim, 1968; Uyeda & Racker, 1965; Bahadur & Verma, 1960; cited by Jones & Greenfield, 1984).

Absolute requirements for calcium are rarely demonstrated (Hughes & Poole, 1989). It is believed to be an important regulator of growth in many micro-organisms (Lloyd, Poole, & Edwards, 1982; Sisken, 1980; cited by Hughes & Poole, 1989) although the mode of action within the yeast cell cycle is still being investigated (Ohya, Ohsumi, & Anraku, 1984). A well-known regulatory effect is the 'triggering' of a range of biochemical processes by an influx of calcium ions either from the external environment or from intracellular stores to an otherwise calcium-free cytoplasm (Harold, 1986; cited by Hughes & Poole, 1989). The influx of calcium does not exert an effect directly but operates through a calcium-binding protein (such as calmodulin (Eilam, 1984; Hubbard, Bradley, Sullivan, Shepherd, & Forrester, 1982; cited by Jones & Gadd, 1990)) which then activates specific enzymes itself thus permitting greater specificity to be achieved. A number of calcium-dependent regulatory proteins have been isolated from yeasts similar to calmodulin (Nakamura, Fujita, Eguchi, & Yazawa, 1984; cited by Hughes & Poole, 1989). The exclusion of calcium from the cytoplasm is not only necessary to prevent intracellular precipitation but also to prevent antagonistic competition with magnesium (See below).

Zinc plays an important but as yet undefined role in yeast morphogenesis during meiosis (Bilinski, Miller, & Girvitz, 1983; cited by Hughes & Poole, 1989) but no true regulatory role has been described. However, as already noted, calcium's effect on the growth of *S. pastorianus* is dependent on the presence of zinc (Lomander, 1965; cited by Densky *et al.*, 1966) and it is therefore possible that zinc exerts a regulatory influence on the incorporation of calcium into membrane structures. Further work on the role of zinc in the control of the morphology of *Candida* species has been carried out (Soll, 1985).

Copper, manganese, cobalt and iron appear to have no known regulatory influence within yeasts but it is feasible that ion-ion inter-relationships exist which may serve to stimulate or inhibit the actions of other ions. For example, copper and

cadmium are more toxic to the yeast together than independently whilst manganese can inhibit their toxicity (Sahinkaya, 1960; cited by Frey, DeWitt, & Bellomy, 1967) or by analogy, the uptake of potassium can be inhibited by the presence of other alkali cations.

Studies on the toxicity of metals on yeast growth and fermentative ability originally focused on the effects of copper (White & Munns, 1951) because of its extensive use in brewing equipment (Frey *et al.*, 1967). However, studies were expanded to other metals (White & Munns, 1951) to categorise poisoning capacities and establish hierarchies of requirements (Meier & Schuler, 1961). Although the studies were conducted in defined synthetic media it was argued that the levels tolerated by the cells would depend on the complexity of the medium and the presence of chelators and sequestrants (White & Munns, 1951). Observations of toxic effects in natural media were carried out (Frey *et al.*, 1967) and it was recognised that definitions concerning levels of inhibition should be based on the concentration of 'free' species within the medium. However, chelators can be used to control the free concentration within defined media and thus prevent undesirable precipitation of the metal (Gaunt, Trinci, & Lynch, 1984; cited by Hughes & Poole, 1989) as well as reducing any toxic effect experienced by the cell (Domek, Lechevallier, Cameron, & M^{c}Feters, 1984; Hutner, 1972; cited by Hughes & Poole, 1989).

Cadmium, mercury, thallium, tin, lead, arsenic and silver are the most common examples of toxic cations although many of the essential transition metals become toxic at elevated concentrations, e.g. zinc, manganese, cobalt and iron. Toxicity is effected by a variety of mechanisms; the most common being either the direct irreversible substitution of essential metals from their binding sites or the indiscriminate binding to bio-polymers which causes haphazard conformational changes. This reflects the view of Williams (1979) that it is the fundamental chemistry of each ion that dictates its role (and therefore toxic capabilities) within the cell. Jones and Greenfield (1984) review toxic metals and their effects.

Sodium can diffuse into cells when present in the medium at elevated concentrations and the cell must expend energy to pump the cation out against the gradient.

Medium copper levels more than 4M can cause irreversible membrane damage resulting in the leakage of potassium that leads to a general loss of viability whilst intracellular levels in excess of cell requirements damages cytoplasmic enzymes but appears not to affect sugar transport mechanisms. Zinc can ameliorate the detrimental effect of copper on the cells (Soumalainen & Oura, 1971; cited by Lievense & Lim, 1982).

Aluminium (Al^{3+}) is recognised as being toxic to microbial cells as it can substitute for magnesium in Mg^{2+}-dependent enzyme systems. Less-than nanomolar quantities of free aluminium effectively compete with millimolar concentrations of magnesium due to the aluminium's greater affinity for the ligands present in such systems (Martin, 1986; cited by Hughes & Poole, 1989). Enzyme activity is not maintained during aluminium substitution as the stronger affinity between the enzyme's ligands implies a reduced rate of ligand exchange that disables the formation of reaction intermediates.

S. cerevisiae can respond to individual toxic ions through the production of specific metallothionenes that can regulate concentration of the offending ion (Christie & Costa, 1984; cited by Jones & Gadd, 1990).

Calcium ions potentiate the membrane damage caused by methyl phenidate (Spoerl, 1971). High concentrations of cations can inhibit cell flocculation in a reversible, time-dependent manner (Stratford & Brundish, 1990).

Physiological Levels of Metal Ions

All equilibria and rates of reaction will depend on the concentration of the free ion although concentrations measured analytically usually represent the total concentration of the ion in question. Several methodologies have been proposed to quantitate the metal ion species within complex media systems under anaerobic

(Callander & Barford, 1983a; 1983b), and aerobic conditions (Pirt & Pirt, 1977; Lie, Hauklie, & Jacobson, 1975; cited by Callander & Barford, 1983a). However, it is recognised that a considerable amount of research is still required to develop analytical techniques capable of ascertaining the distribution patterns of cationic species within the medium (Callander & Barford, 1983b). The complexity of cationic effects on the cells is increased by ion-ion inter-relationships which result in any individual ion's 'optimum' concentration being strongly influenced by the levels of other cations within the medium (Jones & Greenfield, 1984).

Biological essentiality is demonstrated by reducing the concentration of the metal within the medium until the growth of the organism is affected and becomes proportional to the concentration of the cation. However, this method can be prone to contamination problems at the trace levels involved as well as yield misleading results when substitution between metals of similar chemistry occurs.

Optimal media composition of inorganic salts has been determined using chemostat methods (Mateles & Battat, 1974) whilst Nagamune, Endo, & Inoue (1980) used a synthetic medium to assess the impact of glucose and metal ions on yeast physiological activities using variance analysis to show how each ions relative activity is dependent on its ratio to glucose.

Storage and Location of Metal Ions

Separation of ions yielding intracellular ion distribution patterns is shown to enable cells to maintain osmotic gradients; establish membrane potentials; permit an interchange of forms of energy (Serrano, 1988; Goffeau & Boury, 1986; cited by Jones & Gadd, 1990) and afford an extra level of control within the regulation of enzymes and metabolic pathways.

Uptake and Release of Metal Ions

It is of intracellular consequence that cells be able to accurately distinguish types of cations, despite similar ionic radii, (e.g. Na^{+} and $Ca^{2+)}$, by exploiting any differences in their intrinsic chemical properties but it is equally necessary for the cell to be able to ensure an adequate supply of the required metal through active uptake mechanisms. A high degree of selectivity must be present within the binding sites of the cells biomolecules to ensure the placing of a specific cation at the required site whilst simultaneously excluding any competing ions; a situation must also prevail within the membrane transport systems for ions both external to the cell and 'compartmentalized' within the cell. A review of the possible chemical-selectivity strategies and the individual ion-transport systems used by the cell is covered by Hughes and Poole (1989) whilst a discussion of the influx and efflux transport mechanisms through yeast plasma membranes and vacuolar membranes is available (Jones & Gadd, 1990).

Yeasts accumulate potassium very quickly within the cytoplasm, nucleus (Roomans & Seveus, 1976; cited by Hughes & Poole, 1989) and intracellular vacuoles (Okorokov, Lichko, & Kulaev, 1980; cited by Hughes & Poole, 1989) as transported by a complex, though well-defined, system (Rothstein, 1974; cited by Hughes & Poole, 1989). A second system with a much greater affinity for potassium may exist within the cells (Rodriguez-Navarro & Ramos, 1984; cited by Hughes & Poole, 1989). Control of uptake may come in part from the non-competitive inhibition of other ions that bind to a 'modifier' site located on the carrier protein whilst direct competitive inhibition can also affect the kinetics of uptake. The yeast cell can establish transmembrane potassium gradients to generate a driving force for the transport of calcium, magnesium, leucine, glycine, and glucose (Lichko, Okarokov, & Kulaev, 1980; cited by Hughes & Poole, 1989). However, the uptake of potassium requires not only membrane potential but is also believed to be linked to ATP hydrolysis (Tromballa, 1980; cited by Hughes & Poole, 1989) by way of an energy-dependent

electro-neutral K^+/H^+-exchanging ATPase (Malpartida & Serrano, 1981; cited by Hughes & Poole, 1989).

Calcium uptake across most eukaryotic membranes has been reviewed (Silver, 1977; cited by Hughes & Poole, 1989) whilst the system for fission yeast has been described (Boutry, Foury, & Goffeau, 1977; cited by Hughes & Poole, 1989). However, a mitochondrial transport system appears to be absent in yeasts (Lloyd, 1974; cited by Hughes & Poole, 1989). The difference between the calcium requirements of *S. cerevisiae* and other yeasts is reflected by the differences in the K_m values of the uptake systems; 500μM for *S. cerevisiae* and 45μM for *S. pombe* (Jones & Greenfield, 1984). Calcium efflux systems have been described, some of which are energy-dependent (Pitt & Ugalde, 1984; cited by Hughes & Poole, 1989).

Zinc uptake has been widely studied in yeasts where several transport systems exist which show differing selectivities for the metal and hence different sensitivities to competing cations. Mowll and Gadd (1983; cited by Hughes & Poole, 1989) report large differences between the K_m of the zinc transport system in *S. cerevisiae* and that of the system present in *Sporobolomyces roseus* (5mM and 0.09mM respectively).

Manganese uptake is controlled by the intracellular concentration of potassium and ATP with the actual quantities extracted from the medium being very low, similar to iron in this respect, whilst cobalt and zinc uptake are similar in being extremely rapid. All four cations appear to be only taken up from the medium by the yeast cells during the exponential phase of growth.

1.2.3.6. Magnesium Ions in Yeast Physiology

Magnesium is essential for microbial growth (Jones & Gadd, 1990; Hughes & Poole, 1989). It acts as a co-factor for many yeast enzymes (Jones & Greenfield, 1984) including membrane-bound adenyl cyclase (Nurminen & Suomalainen, 1973) and adenosine nucleotides (Slaughter, 1973; cited by Saltukoglu & Slaughter, 1983). It is a structural component of ribosomes (Williams, 1970) and cell membranes (Jones & Greenfield, 1984) and a regulator of cell division in *S. pombe* (Walker & Duffus, 1980).

Reviews of magnesium within higher eukaryotic cells are available (Sigel & Sigel, 1990) although it is acknowledged that much of magnesium's physiological influence remains to be studied (Maguire, 1990). For lower eukaryotes, there is also a shortage of studies concerning the role of magnesium within the cell (Walker, Maynard, & Johns, 1990a & 1990b) although some reviews of metal ions cover magnesium within microbial cells (Hughes & Poole, 1990) and yeast cells (Jones & Gadd, 1990; Jones & Greenfield, 1984). This survey is confined to the role of magnesium within microbial cells, attempting to focus on *S. cerevisiae*, although some references to other yeasts and mammalian cells are made where necessary.

Functions of Magnesium

Calcium and magnesium have traditionally been associated due to their proximity within the periodic table and, as mentioned previously, they are generally involved in structural roles and the transfer of ionic charges. However, there are several differences between them regarding complex formation that will influence their biological function: calcium has a co-ordination number of seven or eight yielding irregular co-ordination geometry in terms of bond angle and bond length whereas magnesium shows a co-ordination number of six that results in a closely regular octahedron. Thus, magnesium-protein complexes would be expected to be less common than the calcium counter-parts owing to the irregular geometries associated with biological molecules (Williams, 1976). Similarly, cross-linking demands such high co-ordination numbers and irregular geometry that it will be a primary feature of calcium rather than magnesium chemistry (Hughes & Poole, 1989). The calcium cross-link is associated with molecules located on the cell surface such as membrane phospholipids-to-proteins (Lewis *et al.*, 1978) whereas the weak magnesium cross-link is associated with tRNA, rRNA and the sub-units of ribosomes. The reversible nature of ionic cross-linking, compared to the more rigid sulphur bridges, is believed to facilitate a rapid response to changing conditions (Williams, 1976).

Magnesium has been shown to stimulate the growth response of microbes to other metals although the growth requirement for magnesium can not be met by other metals (Tempest, 1969; cited by Jones & Greenfield, 1984). However, manganese can replace magnesium in some cellular processes such as cell division (Utkilen, 1984). The action of calcium is known to be antagonistic to the correct functioning of magnesium (Saltukoglu & Slaughter, 1983) and one proposal (Jones & Gadd, 1990) is that the calcium out-competes the magnesium when binding to ATP and nucleic acids because of its stronger binding affinity. Inactivated complexes then predominate (Williams, 1970), causing a situation similar to that described previously for aluminium. The tighter binding and slower dissociation of calcium from a magnesium-binding site on a dephosphorylating phosphoenzyme from mammalian gastric cells is also suggested as a reason for the slower enzyme kinetics (Mendlein & Sachs, 1989).

Magnesium is considered as maintaining the function of many yeast cell constituents by providing a shielding effect to anionic sites, especially concerning membrane integrity and function where its level of incorporation is significantly higher than that of calcium (Jones & Greenfield, 1984). This membrane involvement appears to be related to protein-protein interactions as opposed to the lipid-protein actions defined for calcium (Jones & Greenfield, 1984). The linkage of ATPase to the yeast membrane (Lewis *et al.*, 1978) requires magnesium to aid the reassociation of free ATPase with the membrane (Abrams & Baron, 1968). Magnesium's influence over membrane structure and integrity extends to the control of the exchange of monovalent cations such as K^+/H^+ and Na^+/H^+ (Rodriguez-Navarro & Sancho, 1979) which may, in part, account for the observation that magnesium represses the weight loss of *S. cerevisiae* caused by the presence of sodium undecylenate (Tani, Kuriyama, & Otsuka, 1967).

The conformational folding of large structures such as nucleic acids, which contain many anionic groups, e.g. phosphates, can usually only occur if the negative charges are shielded by magnesium. For example, denaturation of the DNA strand is

associated with the release of magnesium (Wu, Dattagupta, Hogan, & Crothers, 1979; cited by Hughes & Poole, 1989) and yeast transfer RNA can only bind amino acids when magnesium is present (Teeter, Quigley, & Rich, 1981; cited by Hughes & Poole, 1989). Within the tRNA molecule, 'weak' binding is thought to be responsible for general folding effects and association to the ribosomes whilst 'strong' binding is thought to effect more specific conformational changes (Teeter *et al.*, 1981; cited by Hughes & Poole, 1989).

Ribosomes rely on magnesium for the stability of their structure and the interaction has been extensively studied for *E. coli* (Goss, Parkhurst, & Wahba, 1980; Gorisch, Goss, & Parkhurst, 1976; cited by Hughes & Poole, 1989). Changes in the mitochondrial structure of *S. pombe* have been shown to be dependent on the presence of magnesium ions (Walker & Birch-Anderson, 1984; Walker, Birch-Anderson, Hamburger, & Kramhoft, 1982). These changes in mitochondrial structure have been correlated to changes in the respiratory quotient of the cells as a function of magnesium supply (Walker *et al.*, 1982).

Magnesium stimulates yeast fermentation by enhancing the uptake of phosphate from the medium (Nordheim, 1968; Borst-Pauwels, 1967) possibly by the stabilisation of the sub-units of the phosphate transport system (Jones & Greenfield, 1984).

Five classes of enzymes require magnesium as a cofactor (Mahler, 1961; cited by Heaton, 1990) with the largest group of magnesium-activated enzymes being those which effect the transfer of the terminal phosphoryl group from ATP, e.g. kinases, synthetases and phosphatases (Heaton, 1990). The major roles of phosphorylating enzymes within yeast glycolysis, e.g. hexokinase, phosphofructokinase, phosphoglycerate kinase, and pyruvate kinase (Pederson & Gupta, 1979; cited by Hughes & Poole, 1989), and the involvement of other classes of enzymes within the TCA cycle, e.g. pyruvate dehydrogenase, citrate synthase, and succinyl-CoA synthetase, infers significant cellular importance to magnesium. This is supported by

the knowledge that magnesium-requiring enzymes from the other groups are involved in virtually every aspect of biosynthesis (Duffus & Walker, 1985).

There appear to be two mechanisms by which magnesium can activate enzymes: the first involving the formation of an active substrate and the second the formation of an active enzyme. An example of the first mechanism is the binding of magnesium to ATP to yield an $Mg(ATP)^{2-}$ complex. This bonding renders the terminal phosphoryl group more susceptible to a nucleophilic attack from a co-substrate due to the electrophilic nature of the magnesium (Mildvan, 1987; cited by Heaton 1990). An $Mg(ADP)^{-}$ complex appears to be the substrate for creatine kinase (O'Sullivan, Diefenbach, & Cohn, 1966; cited by Heaton, 1990) whilst other studies suggest that magnesium may activate isocitrate, the substrate of isocitrate dehydrogenase (Plaut, Schramm, & Aogaichi, 1974; cited by Heaton, 1990). The second mechanism involves the magnesium binding directly to the enzyme to produce the conformational changes necessary for catalytic activity. For example, enolase (Rose, 1987) binds its full complement of magnesium only in the presence of the substrate, 2-phosphoglycerate, whereupon the enzyme becomes fully active (Brewer, 1981; cited by Hughes & Poole, 1989).

Magnesium ions have been shown to be essential for cell division in the fission yeast *S. pombe* (Walker & Duffus, 1980) using chelating agents and the introduction of cells to Mg^{2+}-deficient media (Lloyd *et al.*, 1982; cited by Hughes & Poole, 1989). In the latter instance, the cells continue to elongate but nuclear division and cell plate formation are inhibited (Walker & Duffus, 1980) which suggests that magnesium deficiency affects events late in the cell cycle. Magnesium deficiency causes a blockage of cell division at the G_2/M boundary of the cell cycle (Walker & Duffus, 1980); a fact that is verified by magnesium shift-up of magnesium-limited cultures causing a synchronization of cell division (Walker & Duffus, 1980). Use of ionophores to artificially reduce the intracellular magnesium concentration accelerates the cell

cycle, indicating a causative rather than a responsive effect for magnesium (Walker, 1986).

Use of synchronized cell populations of *S. pombe* have enabled accurate measurements of the total intracellular magnesium to be made (Walker & Duffus, 1980); the findings revealed that no increase in the magnesium content occurred as the cells elongated. Thus, a steady fall in the intracellular magnesium concentration as a result of the increasing cell size occurs. However, a rapid influx of magnesium occurs immediately before cell division (Walker & Duffus, 1980) which may be responsible for microtubule disassembly (Walker & Duffus, 1983). These cyclic fluctuations of magnesium may act as a transducer of cell size correlating cell growth to mitosis and cell division (Walker & Duffus, 1983).

Magnesium has been implicated in the co-ordination of cellular events (Rubin, 1975) because of its ubiquitous involvement with several major classes of enzymes whose relative activities will be influenced by the availability of the cation (Duffus & Walker, 1985). For example, a possible controlling role for magnesium in the synthesis of RNA has been proposed based on chemostat studies (Tempest, 1969; cited by Jones & Greenfield, 1984) which show how the intracellular magnesium requirement is closely paralleled by changes in the RNA content of the cell as a function of growth rate.

Magnesium depresses the formation of fusel alcohols such as *iso*butanol and D-pentanol (Nordstrom & Carlsson, 1965) whilst total inhibition of growth has been reported to occur at 1M (Jones & Greenfield, 1984).

Physiological Levels of Magnesium

Relative to other essential cations, magnesium is considered as a bulk intracellular species (Hughes & Poole, 1989) based on the concentration at which it becomes limiting for growth (Pirt, 1975). For *Saccharomyces* species, the minimum requirement for growth and fermentation in a batch culture of minimal media has been reported as

1.7mM (Jones & Greenfield, 1984) although Lotan *et al.* (1976) state that the optimal concentration for growth of *S. carlsbergensis* is 8.23mM (200µg/ml). Alternatively, the minimum quantity of magnesium required to produce 100g dry weight when the species is growth-limiting is 0.1 - 0.4g (Hughes & Poole, 1989). However, yield varies with the growth conditions employed (Hughes & Poole, 1989; Saltukoglu & Slaughter, 1983). The optimal range for batch-growth in Saccharomyces species on minimal media has been reported as 2 - 4mM (Jones & Greenfield, 1984) although Nagamune *et al.* (1980) report that cell growth was affected by a magnesium concentration of 7/1000 of the glucose level. Growth-limiting values for YEPD are different again; less-than 0.05mM for *S. pombe* and less-than 0.20mM for *S. cerevisiae.*

A positive effect of magnesium sulphate on yeast growth in *Difco* yeast carbon base was observed (Nordstrom & Carlsson, 1965) in the range 0-1.65mM (0-40mg/L) for sake yeast and 0-4.11mM (0-100mg/L) for a brewer's yeast whilst the supplementing of synthetic media with magnesium sulphate has been found to ameliorate the effects of high osmotic pressure (D'Amore, Panchal, Russell, & Stewart, 1988). Exponential growth was extended, and the decline in the fermentative activity of *S. cerevisiae* slowed, by the use of a high glucose concentration in YEPD medium with 0.5mM Mg^{2+} (Dombek & Ingram, 1986).

Storage and Location of Magnesium

The intracellular concentration of magnesium within yeasts is relatively high compared to other ions, being in the range 3.3mM-20mM (Hughes & Poole, 1989). However, nearly 70% of the total magnesium within *S. pombe* (Walker, 1985) is associated to ATP, ADP, nucleic acids and ribosomes whilst 40% may be enclosed in yeast cytoplasmic vacuoles (Jones & Gadd, 1990; Gadd & White, 1989). Animal cells may have only 0.5-5% as free magnesium (Reinhart, 1988; Flatman, 1984; cited by Maguire, 1990) but microbial cells may be as high as 20mM (Okorokov, Lichko, Kholodenko, Kadomtseva, Petrikevich, Zaichkin, & Karimova, 1975) although other reports (Lusk, Williams, & Kennedy, 1968; cited by Utkilen, 1984) suggest upper limits

of 1-4mM. Polymeric orthophosphates of magnesium (Okorokov *et al.*, 1975) may act to regulate the cytoplasmic concentration of free magnesium (Lichko *et al.*, 1982; cited by Jones & Gadd, 1990; Okorokov, Lichko, Kadomtseva, Kholodenko, Petrikevich, Zaichkin, & Karimova, 1974; cited by Hughes & Poole, 1989) using an ATPase-generated, proton gradient across the vacuolar membrane (Eilam, Lavi, & Grossowicz, 1985; Okorokov, 1985; Nicolay, Scheffers, Bruinenburg, & Kaptein, 1982; cited by Jones & Gadd, 1990). This process does not appear to be used for phosphate transport (Shanks & Bailey, 1990).

Diamond and Rose (1970) showed that several ions, including magnesium, limit the lysis of spheroplasts at 30°C and propose that this reveals the presence of unoccupied ion-binding sites on the membrane as a result of spheroplast formation that could indicate a role for the cell wall in maintaining the ionic environment of the membrane of the intact cell.

Uptake and Release of Magnesium

It has been stated that stringent control of the intracellular concentration of an ion indicates a regulatory function for that ion (Maguire, 1990); this appears to be the case for intracellular magnesium concentration within higher eukaryotes (Maguire, 1990).

Transport systems within the yeast cell are analogous to those found in bacteria (Jasper & Silver, 1977; cited by Hughes & Poole, 1989). Within *S. cerevisiae* there exist magnesium-specific, ATP-dependent, high-affinity carrier systems (Bianchi, Carbone, & Lucchini, 1981) with phosphate apparently acting as the charge-compensator but magnesium may also be transported through the potassium carrier systems as well as competing for the manganese carrier (Rothstein, Hayes, Jennings, & Hooper, 1958; cited by Hughes & Poole, 1989). Cobalt and magnesium requirements in certain yeasts and fungi are closely integrated (Hutner, 1972) as both cations may share uptake systems and intracellular binding sites. The uptake of magnesium by yeasts is dependent on the presence of potassium that is in turn dependent on the presence of phosphate (Bahadur & Verma, 1959). Further work on the characterization

of a magnesium-transport system within *S. cerevisiae* is currently in progress (Maguire, 1990).

Magnesium uptake patterns for wild-type *S. cerevisiae* are available (Bianchi *et al.*, 1981) whilst data for laboratory strains simply details the amount of magnesium removed from a minimal medium at the end of a fermentation. A constant value of 1.1pg Mg new cell^{-1} was obtained above an initial medium magnesium concentration of 0.66mM (16mg/L) (Saltukoglu & Slaughter, 1983). (Maximum uptake according to Netter and Sachs (1961) is 9mM per litre of yeast at a medium concentration of 1.1mM magnesium). The concentration of free magnesium within cells of *S. cerevisiae* appears not to be influenced by the external medium concentration (Okorokov *et al.*, 1975) whilst the overall magnesium content of many eukaryotic cells increases by less than two-fold for a 10,000-fold increase in the medium (Flatman, 1984; cited by Maguire, 1990). The latter case is in marked contrast to the situation for the total calcium of eukaryotic cells (Rasmussen, 1986; cited by Maguire, 1990).

Stephanopoulos and Lewis (1968) described a process of excretion of magnesium and potassium associated with phosphate leakage from cells placed in a glucose solution. The process was believed to be associated with an increase in the permeability of the cell membrane as a normal physiological response to the presence of fermentable sugars. The response was shown to be independent of the osmotic pressure of the medium and the energy derived from fermentation and mitigated by the presence of calcium ions (Lewis & Stephanopoulos, 1967).

1.3. Summary

There is considerable economic interest in the use of yeasts as producers of a wide range of chemical compounds, however, physiological studies on industrial strains are scarce in the literature, although there is an obvious need to be able to control yeast cultures if substrates are to be used efficiently. Control of cultures is difficult and usually involves waiting for a problem to occur before implementing non-specific curative measures that are often too late to be truly effective.

A deeper understanding of the role of the culture environment in the regulation of cell metabolism is seen as a way of exerting direct control over cell cultures both in an immediate response and in a more long-term, predictive manner.

The environmental magnesium status is of prime importance to the cell, fulfilling multiple roles as an enzyme cofactor, a structural agent and a regulator of cell cycle events. These cell requirements justify studies into the influence of magnesium on yeast intermediary metabolism.

1.4. Aims and Objectives of the Thesis

The following represent the main aims and objectives of this study:

a) To characterize the magnesium requirements of *S. cerevisiae* during growth and fermentation within a batch culture and establish the magnesium-concentration at which growth is limited and the influence of magnesium-limitation on the fermentative capacity of the organism.

b) To investigate the physiological states of *S. cerevisiae* over a range of growth rates using a magnesium-limited chemostat and compare the results to those established within batch culture.

c) To study the metabolic behaviour of *S. cerevisiae* when released from magnesium-limitation with especial regard to the influence of regulatory phenomena such as the Crabtree and Pasteur effects.

CHAPTER 2: METHODS

2.1. Batch Fermentations

2.1.1. Fermenter Set-up

Fermentations were carried out in four, 2-litre Life Science Laboratories bioreactors (Model LM2S) operated at a working volume of 1 litre. The vessels were produced in borosilicate glass with a hemispherical base and an external jacket connected to a circulating, thermostatically-controlled water-bath (LSL Ltd., 1985).

Temperature in the vessel was controlled by a microprocessor-based controller that monitored the temperature of the culture by means of a factory-calibrated thermistor located within a blind pocket on the vessel's stainless steel head-plate. The temperature of the culture was periodically checked using a standard mercury thermometer placed in the blind pocket. No discrepancies were ever observed. Set-point for all fermentations was 30^{o}C +/- 0.1^{o}C.

Stirring of the culture was by top-drive agitation with the shaft passing through the head-plate by way of a sealed mechanism that carried the detachable drive motor. Two, 4-bladed turbines were mounted on the shaft at the 0.33 and 0.66 litre marks to ensure maximum possible mixing. Measurement and control of stirring were through a tachometric sensor mounted in the head of the drive motor. Set-point for all fermentations was 250rpm +/- 1rpm.

Aeration of the culture was managed not by a feedback system but by manually adjusting the air-flow controller that measured and restricted the flow of air from the air pump, located in the main control cabinet, to the vessel. The air was always passed through a 0.22 micron filter immediately prior to entering the vessel. Aeration flow-rates were set to supply excess air to the culture, usually at a maximum of 2.5v/v/min.

Vessels were de-ionized as detailed in Appendix 4. The deionization process used has been found to be sufficient (Seiler, 1990) although because magnesium is highly ubiquitous, it is thought that dust particles may be a source of contamination (Sansoni and Iyengar, 1980; cited by Seiler, 1990). Subsequent sterilization of the sealed, empty

vessels was carried out by autoclaving at 121°C, 15psi for 15 minutes with a 'freesteam' time of 10 minutes at the 100°C mark. This procedure was also followed for medium sterilization. After autoclaving, the vessels were re-integrated to the rest of the system and cooled to the required operating temperature.

2.1.2. Medium Preparation

Artificial culture media have already been developed for the study of yeasts. Edinburgh Minimal Medium N°.3 (EMM3) was chosen as the starting-point for these studies as it has been used extensively for work with *Schizosaccharomyces pombe* (Fantes & Nurse, 1977). However, the original medium, EMM2, was designed for propagation studies (Mitchison, 1977) whilst the studies here focus more on respiro-fermentative metabolism. Therefore the starting-level of glucose was increased from the recommended 0.1%w/v to 2.5%w/v. This ensured that fermentative metabolism occurred because of the Crabtree Effect, as it was felt that the studies would be hindered by imposing strictly anaerobic conditions (Tyagi, 1984).

Analar-grade reagents were used throughout, as was 18 MegaOhm (ultra-pure) water that had been passed through a 0.22 micron filter. Stock solutions of the major constituents were made up volumetrically and the necessary volume of each added to a 5-litre, grade-A volumetric flask, which was then made up to the mark. Details are given in Table 2.1 below.

Table 2.1: Procedure for the preparation of modified Edinburgh Minimal Media.

Constituent (Formulae)	**Stock Solution** (Grams of constituent added to litres of water)	**Dilutions** (Litres of stock solution added to 5L volumetric flask)	**Medium Levels** (Resultant concentration of constituents)
Glucose $C_6H_{12}O_6$	125 g in 0.5 L	0.5 L	25 g/L

Nitrogen NH_4Cl	25 g in 0.25 L	0.25 L	5 g/L
Phosphate Na_2HPO_4	071 g in 2 L	0.2 L	1.42 g/L
Magnesium $MgCl_2.6H_2O$	20. 3 g in 1 L 100mM Mg stock solution*	None added at this stage	Basal contamination
Salts $KC_8H_5O_4$ $CaCl_2.2H_2O$ KCl Na_2SO_4	x g specified salt in 2 L 153 g 0.5 g 50 g 15 g	0.2 L	x g/L 3.06 g/L 0.01 g/L 1 g/L 0.3 g/L
Trace Elements H_3BO_3 $MnSO_4.H_2O$ $ZnSO_4.7H_2O$ $FeCl_3.6H_2O$ $H_2MoO_4.H_2O$ KI $CuSO_4.5H_2O$ Citric Acid	x g specified trace in 1 L 0.05 g 0.04 g 0.04 g 0.02 g 0.016 g 0.01 g 0.004 g 0.1 g	0.05 L	x g/L 0.0005 g/L 0.0004 g/L 0.0004 g/L 0.0002 g/L 0.00016 g/L 0.0001 g/L 0.00004 g/L 0.001 g/L
Vitamins Inositol Nicotinic Acid Ca Pantothenate Biotin	x g specified vitamin in 0.5 L 5 g 5 g 0.5 g 0.005 g	0.005 L	x g/L 0.01 g/L 0.01 g/L 0.001 g/L 0.00001 g/L

* Refer to Appendix 5.

Sterility was achieved by autoclaving the specified volumes of the stock solutions and allowing them to cool before combining volumetrically, thus ensuring no adverse reactions such as precipitation or caramelization occurred. The vitamin stock solution was not autoclaved but instead passed through a 0.22 micron filter. The 5-litre volumetric was sterilized by rinsing with 50ml of 70%v/v ethanol for at least 30 minutes before use with a rinse of 100ml of autoclaved, ultra-pure water to remove any residual ethanol. All other glassware was similarly treated if unsuitable for autoclaving, with the exception of the Bijou bottles used temporarily to hold samples. These were sterilized by placing in a 160^{o}C oven for 16 hours. Plastic Eppendorf micro-centrifuge tubes were sterilized by autoclaving as above, as were normal centrifuge tubes.

One-litre volumes of the medium were dispensed aseptically into the cooled vessels and a further equilibration time of 2 hours allowed during which time aeration was set at maximum to saturate the fermenters prior to inoculation. Aeration was always turned off immediately prior to inoculation. At this point, specific volumes of the sterile magnesium stock solution were added aseptically to the vessels to set the magnesium concentrations at the desired test levels. The remaining litre of medium was used both for analytical purposes and as culture medium for the inoculum as required.

2.1.3. Inoculum

The yeast employed in all experiments was an industrial strain of *Saccharomyces cerevisiae* (Meyen ex-Hansen, 1883; Jensen, 1990) conforming to the usual characteristics of baker's yeast as provided by Mauri Products Limited to British Petroleum's Chemical Division (Hull Works). The yeast was originally supplied to the Hull Works as a 1Kg block of compressed wet yeast, from which a pure culture for these experiments was obtained as follows: a sample taken from the centre of the block, using a sterile wire loop, was streaked onto a Sabouraud Dextrose Agar (SDA) plate, which was then incubated overnight at 30^{o}C. A loopful taken from a single colony on the SDA plate was then streaked onto a Wallerstein Laboratories Nutrient (WLN) agar

plate containing 0.004g/L Actidione (a.k.a. Cyclohexamide). After incubation, cells from a single white colony on the WLN plate were then sub-cultured onto 10ml SDA slopes within Universal bottles and incubated at 30°C for 24 hours, before storing at 4°C for a maximum of ten days before repeating the sub-culture.

To generate a suitable inoculum for the vessels, the following procedure was executed in duplicate: 10ml of sterile water were added to an SDA slope of the yeast to produce a cell suspension, which was aseptically transferred to 100ml of medium, known as the primary inoculum, contained in a screw-top flask. This flask was incubated in an orbital shaker for 12 hours at 30°C, 180rpm, whereupon the entire contents were added to 400ml of fresh medium, known as the secondary inoculum, contained in a second screw-top flask that was incubated as above for 10 hours before cell harvesting. The 500ml of secondary inoculum was divided into 100ml volumes that were individually centrifuged at 4000rpm for 10 minutes. The cell pellet was re-suspended in 20ml of sterile water - pre-warmed to 30°C - and shaken vigorously before being re-centrifuged. A second re-suspension in 20ml of pre-warmed media provided the inoculum for the main fermenting vessels. This procedure ensured the removal of interstitial and loosely-bound surface magnesium.

The magnesium levels of both the primary and secondary inocula were kept equal, with their absolute values being determined by the nature of the experiment. For the preliminary batch experiments the magnesium levels were kept in estimated excess of the yeast's requirements at 2mM.

2.1.4. Sampling Regime

Pre-inoculation and post-inoculation samples were always taken to check the actual starting magnesium levels, although other parameters were also checked. Two 3ml samples were normally taken every 4 hours by way of a 5ml sterile plastic syringe. This was attached to a three-way non-return valve connected to the sampling port of the vessel. The first 5ml extracted were discarded to ensure sampling from within the bulk of the medium and not from within the stand-pipe of the sampling port. Each sample

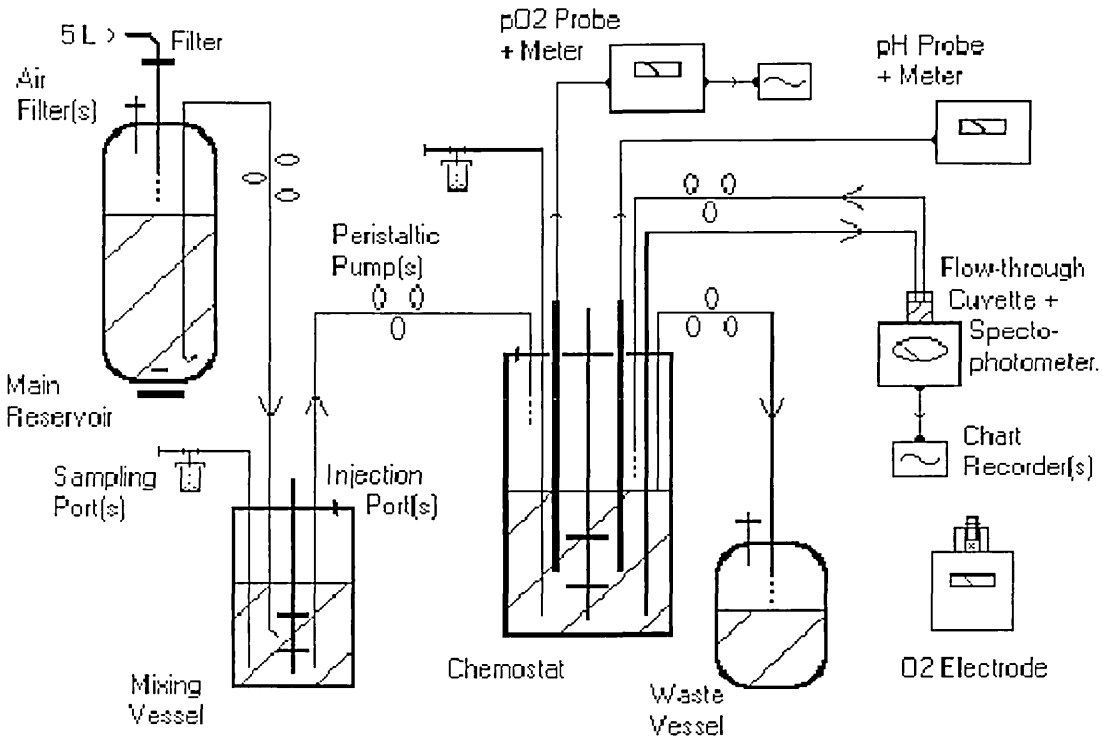
was transferred to a clean, sterile Bijou bottle from which a 2ml volume was measured into a clean, sterile Eppendorf tube and micro-centrifuged at 13,000rpm for 3 minutes, whereupon the supernatant was decanted off the cell pellet into a fresh Eppendorf and frozen to await analysis. This cell pellet was discarded. The Bijou bottle was sealed and the remaining 1ml of sample ultra-sonicated for 4 minutes to separate any cell clumps before cell number determination. The above procedure supplied adequate amounts of test material for most of the analyses, with the exception of dry weight determinations (section 2.3.3) which required two, 10ml samples to be drawn separately. This dried cell pellet was then used for cellular magnesium determinations. (section 2.3.9).

2.2. Continuous Fermentations

2.2.1. Chemostat Set-up

A chemostat was set up by adapting the LSL bioreactors to continuous culture as shown in Figure 2.1 below. The chemostat is a homogeneous single-stage stirred fermenter according to Herbert (1964) and had an operating volume of 800ml as maintained by a pumping-out mechanism (Brown, 1990).

Figure 2.1: Schematic diagram of the chemostat used during continuous fermentations



Aeration levels were controlled as for the batch system although a pO_2 probe was inserted through the head-plate to monitor dissolved oxygen levels. The difficulties of obtaining complete exclusion of oxygen during anaerobic chemostat studies and the associated complications of artificially meeting the yeasts sterol requirements were viewed as justification for carrying out self-regulating, micro-aerophilic studies. Fermentation under aerobic, glucose-repressed conditions is as effective as under anaerobic conditions (Schatzmann, 1975; cited by Fiechter *et al.*, 1981).

Originally, an O_2 electrode was set-up in a manner analogous to the flow-through cuvette; unfortunately this caused too many problems and was abandoned, the option being to transfer a quantity of the culture to the electrode at pertinent times (section 2.3.7).

Sterility was achieved by autoclaving the separate parts as described for the batch fermentations.

2.2.2. Medium Preparation

Medium preparation was as described for the batch fermentations with the following adaptations: 1) The magnesium was added to the medium within the 5-litre volumetric (except where indicated otherwise in individual experiments) prior to making up to the mark in order to minimize the risk of contamination and 2) the 5 litres of fresh medium were introduced to the main reservoir through a Sartotius membrane-filter (Model SM16275) comprising a coarse pre-filter and a 0.22 micron microbiological filter.

2.2.3. Inoculum

The inoculum was prepared (in duplicate) using 10ml of medium to create a cell suspension used to inoculate a screw-top flask containing 90ml of medium. This flask was then incubated in an orbital-shaker for 12 hours at $30^{o}C$, 180rpm before its contents were transferred directly to the chemostat. The inoculated chemostat was allowed to run in batch mode until the cells reached the late exponential phase as determined by changes in optical density and changes in cell density. This took approximately 9 hours, whereupon the influx pumps and efflux pump were started at their lowest rate to initiate a continuous culture, which was then permitted to stabilize for a minimum of four chemostat volumes before any sampling.

2.2.4. Sampling Regime

Samples were physically removed from the chemostat according to the methodology described for batch fermentations (section 2.1.4). The normal sampling regime was to remove a sample of a given dilution rate, re-equilibrate for four chemostat volumes, and then re-sample, after which the dilution rate was altered. However, pulsing and spiking experiments had slightly different regimes detailed in their respective chapters. Processing of samples was identical to batch fermentations with the exception of an extra 3ml volume taken for the O_2 electrode. The mixing vessel provided baseline samples.

2.3. Analyses

A range of analytical methodologies was applied throughout the three types of studies, all of which are specified below. The specific combinations used for individual experiments are detailed in the relevant sections as are any deviations from these normal procedures. All glassware used in the preparation of standards was Grade-A and had been rinsed in ultra-pure water before use, or where necessary de-ionized by the procedure detailed in Appendix 4. Analar or HPLC-grade reagents were used in the preparation of all standards and test solutions.

2.3.1. Cell Numbers

Cell numbers were determined using a Model D: Industrial Coulter Counter (C.C.). Calibration of the C.C. was carried out prior to any given experiment. The accuracy of the C.C. was checked using a Neubaur haemocytometer (Weber BS 748) in conjunction with a phase-contrast microscope. The two methods were in good agreement except during the early to mid-exponential phase of growth when it was found that an undercounting of total numbers occured with the C.C. due to cell aggregation. To minimize this effect, samples were ultra-sonicated in a Camlab Trans-sonic sonicating water-bath (Model T310) before dilution, to ensure adequate dispersal of the clumps. Standard procedure involved sonicating the 1ml sample for four minutes, diluting a suitable volume into 20ml Isoton 2 and carrying out a count using a 100μM orifice-diameter probe. Dilutions ensured that counts lay within the C.C.'s optimum range of 5×10^3 - 2.8×10^4 counts.

2.3.2. Size Distribution

Size distributions of the cell population were also ascertained using the C.C. following established methodology (Johns, 1987). Mean cell volumes (m.c.v.'s) were calculated from the raw data.

2.3.3. Dry Weight

Established methodologies were used as the starting point for dry weight determinations. However, several adaptations were implemented with the resulting procedure being as follows:

Two, 10ml volumetric flasks filled with samples taken from the fermenter were emptied into two, pre-weighed and pre-labelled, centrifuge tubes along with 1ml washings. The tubes were centrifuged at 4000rpm for 10 minutes, whereupon the supernatant was decanted off and the cell pellet resuspended in 10ml ultra-pure water before being re-centrifuged. After decanting of the washing, the cell pellets within the tubes were air-dried at 50^{o}C for 48 hours before cooling in a dessicator for one hour immediately before weighing on a four-place decimal balance.

Where possible, this procedure was executed using 50ml samples as measured volumetrically.

2.3.4. Ethanol

A Perkin-Elmer Gas Chromatograph (GC 8310) was used in conjunction with a 'Poropaq Q' packed column to measure the ethanol concentration of the fermented medium. The technique is well established with the specific methodology used as detailed in Appendix 1. The Flame Ionization Detector (FID) was calibrated by establishing response factors for the internal standard (Iso-propanol) and the analyte (Ethanol) relative to Heptane, the recognized industrial standard (Thorne, 1988), with Nonane serving as a solvent whose retention time was greater than these compounds. A series of test solutions containing the internal standard and analyte was used to check accuracy, retention times and the linear range.

2.3.5. Glucose

A Bio-Rad HPLC pump (Model 1330) was used in conjunction with a Bio-Rad column (Aminex Ion Exclusion HPX-87H for Organic Acids) and Bio-Rad Refractive Index detector for the separation and detection of fermentable sugars and fermentation

products. Operating conditions are specified in Appendix 2. Calibration of the Refractive Index (RI) detector was made using two solutions of external standards containing the compounds of interest to generate an external standards file referenced by the computer on all subsequent runs. The first solution was at 0.1%w/v and the second at 0.2%w/v.

2.3.6. pH

No suitable pH probe was available during the batch fermentations and therefore only the initial and final pH's could be measured. These were measured using a Corning 400 Series pH meter calibrated to two standard solutions, pH = 4 and pH = 7.

2.3.7. O_2 Uptake

A Rank Brothers dissolved oxygen electrode system was used to measure the oxygen uptake rate of the entire cell population as detailed by Johns (1987). The temperature of the electrode was held constant at $30^{o}C$ using an LKB circulating water-bath (Model 2209).

2.3.8. Magnesium Concentration (Medium)

A Perkin-Elmer Atomic Absorption Spectrophotometer 1100B (AAS) provided all measurements of magnesium. Appendix 3 details the operating conditions used throughout as arrived at following manual procedure. The use of the high-sensitivity 285.2nM resonance line was found to be a disadvantage as at higher magnesium concentrations, dilution of the sample was required and therefore the use of the less-sensitive 202.5nM line may have improved the accuracy of the measurement (Seiler, 1990).

Calibration was carried out using a series of standards to establish a standard curve, referenced by the AAS software on all subsequent runs. Standards were made up as follows: Mg ribbon had surface oxides removed; abrasively using emery paper and chemically using 5%v/v conc. HCl. One gram was dissolved in 20ml of 10%v/v

concentrated HNO_3, whereupon the solution was transferred, with washings, to a 1-litre volumetric and made up to the mark to yield a 1000ppm stock solution. A hundred-fold dilution of this stock solution yielded a working solution of 10ppm that was used to produce a series of standards covering the linear range of the AAS.

In the detection of magnesium using AAS, no interferences from sodium, potassium, calcium or phosphate are observed (Allan, 1958; Belcher and Bray, 1962; McBride, 1964; Slavin, 1967 respectively; cited by Seiler, 1990), however, interferences from high concentrations of aluminium or silicon can occur (Humphrey, 1965; Harrison and Wadlin, 1969; cited by Seiler, 1990) although it is not believed these elements were present at significant levels as Analar reagents were used throughout.

2.3.9. Magnesium Concentration (Cells)

The dried cell pellets from the dry weight determinations were resuspended in 2ml water and then hydrolysed by the addition of 2ml concentrated sulphuric acid at room temperature for 24 hours followed by a 10 minute boil to completion. The hydrolates were transferred to 25ml volumetrics with washings and made up to the mark, whereupon the solution was aspirated directly into the AAS where possible or diluted further where not.

The main aim of sample preparation for the AAS is to create a homogenous solution with the removal of the biological matrix being necessary to ensure that the viscosity and surface tension of the samples are as similar as possible to the standards used during calibration. The acid extraction technique employed within these studies releases the magnesium from its binding sites without significantly altering the matrix although the organics may act as a fuel for the flame (Seiler, 1990).

2.3.10. Specific Gravity

Specific gravity measurements were taken at the start and end of all molasses fermentations using a Zeal hydrometer and a 100ml measuring cylinder. 'Tapping' and 'twirling' ensured no air bubbles remained within the cylinder.

The following techniques were applied solely to the chemostat:

2.3.11. Optical Density

The flow-through cuvette provided with the Cecil Series-2 spectrophotometer (Model CE 373) was used to continuously monitor the optical density of the culture at 540nM. Data output was stored on an LKB flat-bed chart-recorder. Zero optical density was set on the circulating, uninoculated medium.

2.3.12. pO_2

An Ingold O_2 sensor (Model E-O_2-12-CH) coupled to a Unilink O_2 meter (Model 703P) provided data on levels of dissolved oxygen, which were continuously recorded on an LKB flat-bed chart-recorder. Calibration of the probe took place in situ after equilibration, and involved setting the upper limit of 100% air-saturation of the media at 30^{0}C by aerating the chemostat for 15 minutes, followed by setting the lower limit of 0% air-saturation by purging the chemostat with nitrogen gas for 15 minutes. This procedure was repeated.

2.3.13. pH

An Ingold pH probe (Model 465) connected to a Whatman pHA 220 meter permitted continual monitoring of culture pH. Calibration was made before autoclaving using the two solutions used to calibrate the Corning electrode. This permitted an external check to ensure no loss of calibration had occurred.

2.3.14. Extracellular Magnesium Concentration (Feed)

Sampling for extracellular magnesium concentration was identical to the regime described for the chemostat (section 2.2.4) whilst analysis was as described for medium magnesium (section 2.3.8).

CHAPTER 3: RESULTS

3.1. Batch Fermentations

3.1.1. Magnesium Requirements within Minimal Media

3.1.1.1. Introduction

The literature reviewed by Jones and Greenfield (1984) indicates a broad range of values for the exogenous magnesium requirements of *S. cerevisiae* applicable to many types of culture conditions. The data available is not of a detailed nature and is of limited use in predicting the influence of medium magnesium levels on population behaviour. The optimal concentrations of magnesium [2-4mM] recommended (Jones and Greenfield, 1984) appear to have been derived empirically from batch systems although subsequent experimental data from continuous culture studies caused a downward revision of the optimum levels to 0.5mM (Jones, 1986).

Experiments studying the exogenous magnesium requirements of a culture of *S. cerevisiae* within a batch minimal-medium system were carried out to provide a characterization of the influence of magnesium on the growth and fermentative capacity of the organism and to establish the magnesium concentration at which growth becomes limited. This characterization was considered necessary not only to extend the information available concerning the responses of yeasts to inorganic cations (Rose, 1987) in terms of uptake patterns and their association with other fermentation parameters but also as a way of establishing the operating range for a magnesium-limited chemostat. The establishment of μ_{max} and K_S is prerequisite to studies in continuous culture where they serve as reference points that enable the comparison of experimental data to theoretical curves.

Hence, the effect of the initial magnesium concentration within the medium on the fermentative and respirative parameters of the cell population was studied for a broad range of concentrations in order to establish where growth-limitation and non-limitation occur as well as to delineate the relevant magnesium uptake patterns.

3.1.1.2. Method

The methodology for the fermenter set-up is detailed in section 2.1.1; the medium preparation in section 2.1.2; and the inoculum preparation in section 2.1.3. whilst the procedure for the analyses of samples is available in section 2.3.

The initial medium magnesium concentrations within each fermenter were selected to cover two ranges devised to represent the information within the literature; i.e. 0-500μM and 1000-4000μM. Appropriate volumes of the 100mM magnesium stock solution were added to the 1L of medium within each fermenter to yield the following test concentrations: 13, 55, 71, 120, 142, 347, 496μM within the first range and 963, 1347, 1656, 2006, 2386, 2587, 2736, 3007, 3829μM within the second range.

3.1.1.3. Results and Discussion

The results detail several directly measured fermentative and respirative aspects of the batch growth of cell populations of *S. cerevisiae* within a minimal medium containing various concentrations of magnesium. Also detailed are several derived parameters calculated from the directly measured results.

For the first range of magnesium concentrations detailed above, (section 3.1.1.2), the effect of the initial magnesium concentration present within the medium on the measured fermentation parameters (cell concentration, ethanol, glucose, and magnesium levels) is shown in Figures 3.1, 3.2, 3.3, and 3.4 respectively. Results for the second range of initial medium magnesium concentrations are not shown as no easily discernible differences existed between the fermentation parameters of the final value of the first range of magnesium concentrations (496μM) and the respective fermentation parameters of the entire second range of magnesium concentrations.

However, the results from the second range of concentrations are included for completeness in the graphs for the derived fermentation variables; such as the specific growth rate and ethanol productivity, the biomass and ethanol yields (glucose and magnesium), and the ethanol production rate and glucose uptake rate; which are shown in Figures 3.5a, 3.6, 3.7, 3.8, 3.9, 3.10, 3.11 and 3.12 respectively. For the purposes of

presentation, the results for the highest magnesium concentration (3829μM) are not shown. They are not significantly different from the other data shown. Similarly, the results for the biomass and ethanol yield (glucose) are only detailed upto the 496μM concentration as the inclusion of the data for the higher concentrations would obscure the trend in the graphs at the lower concentrations.

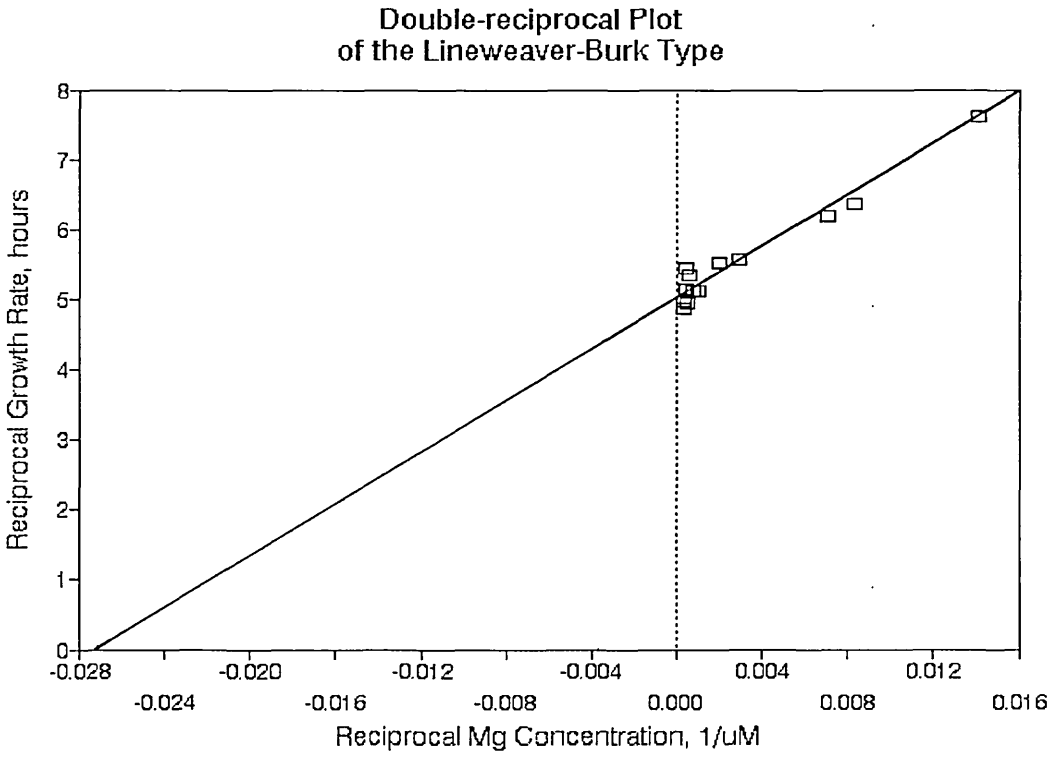
From Figure 3.1, it can be seen that as the initial exogenous magnesium concentration is increased, the characteristic growth curve of a batch culture occurs over an increasingly shorter time. For example, the time taken to reach the stationary phase for an initial magnesium concentration of 55μM is 85hrs (not shown) whereas stationary phase is reached after only 18hrs when a magnesium concentration of 496μM is used.

For all the initial magnesium concentrations tested, the stationary phase cell concentrations were broadly similar, with only the cells growing at the lowest value of 13μM not achieving any significant increase from their inoculation levels. This agrees with the results of Saltukoglu and Slaughter (1983) who found the same level of increase in cell number for initial magnesium concentrations of greater than 247μM. They also noted that at an initial magnesium concentration of 38μM "insufficient growth (occurred) for accurate measurement".

The length of the lag phase depreciates considerably whilst the slope of the exponential phase becomes steeper as the initial concentration of exogenous magnesium is increased. It is these two factors that contribute to the time at which stationary phase is achieved. The results indicate that the magnesium concentration of the medium is affecting the rate of cell growth whilst appearing not to influence the populations total growth. This can be seen more clearly in Figure 3.5a where the hyperbola indicates a Monod relationship between the specific growth rate of the cells and the medium magnesium concentration. From this saturation curve, a maximum growth rate (μ_{max}) of 0.196hrs^{-1} is reached at an initial medium magnesium concentration of 625μM whilst K_S is found to be equal to 40μM. Shkidchenko (1977) found the K_S of *C. utilis*

to be 15μM (0.00037g/L) whilst the K_S of *E. coli* is known to be 31μM and *Rhodopseudomonas capsulata* equal to 55μM (Jasper and Silver, 1977; cited by Hughes & Poole, 1989). Bull (1974) recommends the use of a Lineweaver-Burk type double-reciprocal plot for the accurate determination of the maximum growth rate and the K_S value. Figure 3.5b below is used for this purpose.

Figure 3.5b: A double-reciprocal plot of Growth Rate versus Magnesium Concentration (Figure 3.5a) of the Lineweaver-Burk type



From this graph, μ_{max} is found to be equal to 0.200hrs^{-1} and K_S equal to 35.7μM. This value for μ_{max} is compared to growth rates obtained under different culture conditions (results not shown) whereby the organism grown at a non-limiting concentration of magnesium (2000μM) with only 1%w/v glucose and mild aeration had a growth rate equal to 0.262hrs^{-1} whilst the growth rate for a non-magnesium-limited culture at 1%w/v glucose in the presence of excess oxygen was equal to 0.329hrs^{-1}. The differences observed between the two cultures at 1%w/v glucose reflect the

influence of an increased supply of oxygen whilst the differences in growth rates observed between the 1%w/v and 2.5%w/v glucose cultures possibly arise from the restrictions of the Crabtree effect.

Saltukoglu and Slaughter (1983) obtained a mean generation time (MGT) of 5.9hrs that approximates to a specific growth rate of $0.124hrs^{-1}$ (assuming MGT = Doubling Time) for a magnesium concentration of 206μM (and a concomitant calcium concentration of 70μM; the concentration of calcium within this medium being 68μM) within a minimal medium containing a respiratory-repressive level of glucose (8%w/v). This is in keeping with the growth rate curve of Figure 3.5a.

That no discernible growth occurred at the lowest magnesium concentration infers that the saturation curve does not pass through the origin. This is in keeping with current thinking concerning the idea of maintenance energy whereby there will be sufficient magnesium present to maintain cell viability within the population but not enough to permit growth to occur. In hindsight, a test of cell viability within the population should have been carried out. The unanswered question is whether the population of cells within the culture containing the lowest magnesium concentration (13μM) would have eventually, given sufficient time, achieved a population density similar to the other magnesium concentrations although this is highly unlikely.

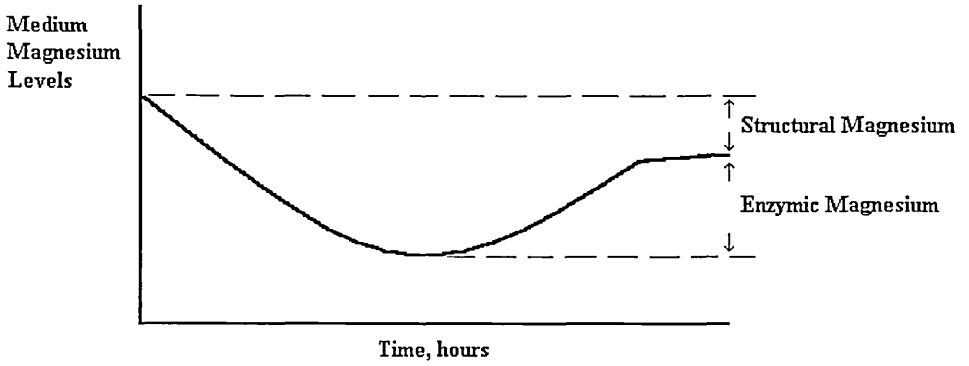
Changes to the ethanol levels within the cultures show a trend similar to the increase in cell concentrations. For all the magnesium concentrations, a final ethanol concentration of approximately 1%w/w was eventually reached; the exception being the culture with an initial magnesium level of 13μM that did not reach higher than 0.25%w/w even after 85hrs. Again, the major difference between the cultures was the amount of time required to reach their final values; for example, the 55μM culture took 72hrs to reach its final concentration whereas the 496μM culture took only 20hrs. These differences are more clearly seen in Figure 3.6 where ethanol productivity is limited by an initial medium magnesium concentration below 625μM.

The trends of the removal of glucose from the medium are in keeping with the growth curves observed. The main difference between cultures is the time taken to exhaust the medium of glucose with the range of times exhibited being similar to the range observed for ethanol production; for example, the 55μM culture takes 72hrs and the 496μM culture takes 20hrs. Again, for all values of the initial magnesium, all the glucose is eventually used with the exception of the 13μM culture that did not manage to remove all the glucose from the medium even after 85hrs (not shown).

Figure 3.4 shows the magnesium uptake patterns from the medium by the various yeast populations. For the cultures in the three highest initial magnesium concentrations, the pattern of uptake is a progressive removal of the magnesium from the medium followed by its gradual release back into the medium. Several aspects of this pattern are noteworthy: the level of magnesium within the medium never returns to its original value but rather achieves a level of some 80-90% of the original concentration; the minimum value for each curve becomes sharper as the initial magnesium concentration increases indicating an increased rate of uptake; the occurrence of the minimum value coincides with the onset of the stationary phase and the exhaustion of the glucose from the medium. (Refer to Table 3.1 below).

The magnesium uptake patterns are in accord with the known roles of the ion in so far as the majority (80-90%) of the total magnesium present appears to be used for non-structural, i.e. co-factor roles inferred by its return to the medium whilst the 10-20% not returned to the medium would presumably have become incorporated into the cell material of the new biomass. This hypothesis is represented schematically in Figure 3.13 below.

Figure 3.13: Proposed utilization of medium magnesium by the cell culture



The lower initial magnesium concentrations show similar uptake patterns although they are not so distinct and easily seen as the uptake patterns of the higher magnesium concentrations. The lowest value (13μM) shows no noticeable uptake pattern at all whilst the second lowest value (55μM) shows a no real removal of magnesium from the medium over the first 36hrs of the culture with uptake not fully beginning to occur until 42hrs into the fermentation. This pattern of magnesium removal is repeated in the 71 and 120μM cultures with the onset of removal beginning earlier (24hrs and 18hrs respectively) with the patterns becoming more similar to those described above for the three higher values of initial magnesium. Hence, the magnesium uptake patterns do not appear to be inflexible but rather they change as a function of the initial magnesium concentration present within the medium.

As already noted above, the occurence of the minima within the medium magnesium for all values of initial magnesium except the lowest value (13μM), appear to be coincident with the onset of the respective stationary phases for the six cultures. This is more clearly demonstrated in Table 3.1.

Table 3.1: Coincidence of stationary phase fermentation parameters with the removal of magnesium from the medium as observed from Figures 3.1, 3.2, 3.3, and 3.4.

Initial Magnesium Concentration within the medium (μM)	Fermentation Time required for medium magnesium to reach a minimum (Hrs)	Fermentation Time required for parameter to reach stationary phase value (Hrs) [Offset between time of minimum magnesium and stationary phase (Hrs)]		
		Cell Concentration	Ethanol Concentration	Glucose Concentration
13	?	SPNR	SPNR	SPNR
55	60	85 [25]	72 [12]	72 [12]
71	39	42 [3]	46 [7]	46 [7]
120	33	34 [1]	36 [3]	36 [3]
142	30	30 [0]	30 [0]	30 [0]
347	22	24 [2]	25 [3]	25 [3]
496	16	18 [2]	20 [4]	20 [4]

where SPNR = Stationary Phase Not Reached

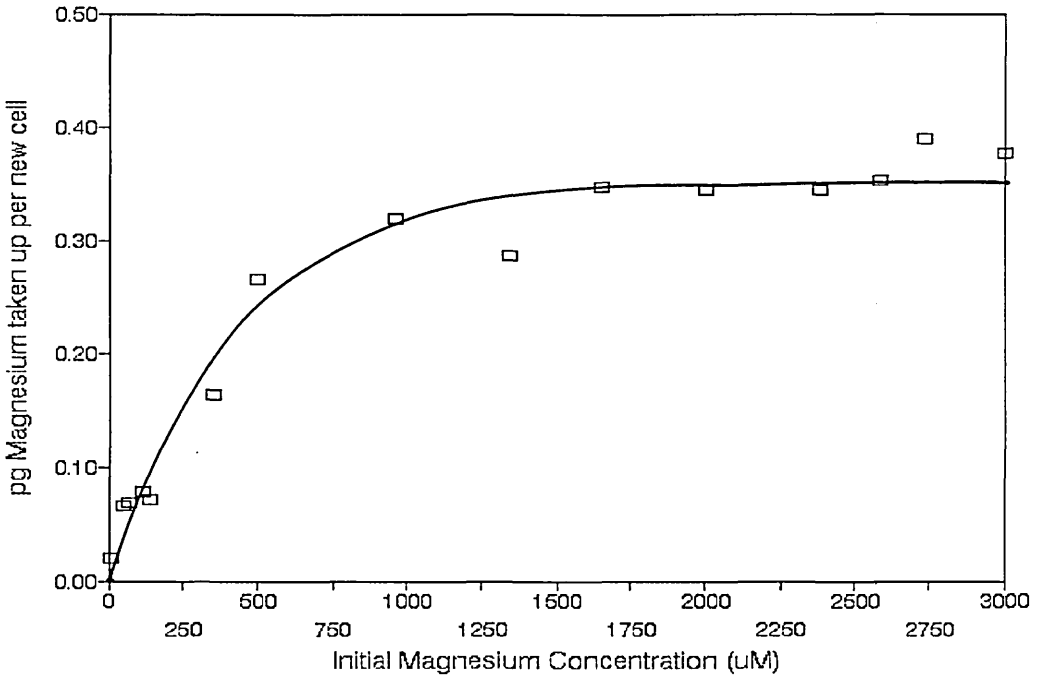
Table 3.1 highlights the slight differences in the time at which the various stationary phase values of the respective fermentation parameters for the different cultures are achieved. Generally, the glucose is depleted at the same time as the ethanol achieves its highest concentration; the cell populations reach their stationary phase values slightly ahead of the glucose depletion or cessation of ethanol production; and magnesium's removal from the cultures occurs ahead of the stationary phase values for the cell concentration, glucose depletion or ethanol production. The latter aspect is of interest as it is in keeping with the known magnesium requirements of intermediary metabolic enzymes within the cell and confirms the close relationship between glucose

utilization and fermentative metabolism. That the magnesium release appears to start before the attainment of plateau levels for the fermentation parameters may reflect magnesium's multiple functions within the cell and may represent the slowing down of yeast glycolytic activity and hence a reduced magnesium requirement by the pertinent phosphorylating enzymes. Similarly, maintenance of a triggering intracellular magnesium concentration will become less important to the cell as division becomes limited by other, unidentified factors and the time difference simply represents the ease with which magnesium can be released from the cell as opposed to the complexity of cell material manufacture.

Figure 3.7 details the relationship between the biomass yield from the glucose and the initial medium magnesium concentration. The graph shows a similarity to the other derived variables, although the rise in biomass yield as a function of magnesium concentration is much sharper, resulting in a leveling out of yield by 75μM. This would infer that the absence or presence of magnesium is more important than the actual magnesium concentration itself; an inference in keeping with the results of Saltukoglu and Slaughter (1983).

To test this, the results were recalculated according to the method used by Saltukoglu and Slaughter (1983) and a graph of the quantity of magnesium taken up per new cell is presented in Figure 3.14 below. Included for completeness are results from the second range of initial magnesium concentrations tested.

Figure 3.14: Quantity of magnesium taken up per cell from the medium as a function of the initial magnesium concentration.



The trend is very similar to the results presented by Saltukoglu and Slaughter (1983) although the actual plateau amounts are only a third of the level attained by those authors. The main identifiable difference is that the glucose levels were significantly higher in the experiment of Saltukoglu and Slaughter (1983). The conclusion is that although the yeast takes up a constant amount of magnesium per cell (Saltukoglu and Slaughter, 1983) the actual amount will be in proportion to the level of glucose present in the medium. The initial magnesium concentration where the plateau occurs is approximately 800μM which comparable to a value of 825μM estimated from the results of Saltukoglu and Slaughter (1983).

Fiechter, Kappeli, & Muessdoerffer, (1987) have indicated that a biomass yield of 0.15g dry cell weight (g glucose)$^{-1}$ for *S. cerevisiae* is possible during oxygenated batch cultivations. The biomass yield obtained within this set of experiments is again only a third of this value and probably reflects the respiratory limitation imposed on the

cells by the excess of glucose coupled with the absence of aeration. Although not shown, the yield did not change in the second range of magnesium concentrations studied.

It is noteworthy that the cells cultured at the lowest magnesium concentration did increase their biomass despite not managing to increase their cell numbers. This would infer that the cells were not only still alive within the culture at 85hrs but also metabolically active; an assumption that is supported by the observation that glucose was being consumed and ethanol produced by this culture. A possible explanation for the lack of cell division occurring within the population is the hypothesis forwarded by Walker and Duffus (1980) who proposed that for cell division to be initiated within *S. pombe* a critical intracellular concentration of magnesium must be reached. It is therefore possible that the contaminant levels of magnesium present within the culture were insufficient to permit the cells to accumulate the proposed 'triggering' concentration. It is not suggested that this scenario is complete as it is known that for the initiation of cell division, growth to some critical size is required (Johnston Ehrhardt, Lorincz, & Carter, 1979) and the cells here are perhaps not growing sufficiently. An investigative experiment would be to repeat the fermentation at the contamination levels of magnesium but use less inoculum. This would effectively lower the competition for the available magnesium by increasing the magnesium-to-cell ratio and hence allow the inferred intracellular triggering concentration of magnesium to be reached. The expected result would be to observe at least one doubling of the cell population. It is known that cells transferred from a magnesium-complete inoculum to a magnesium-deficient medium usually manage a doubling step before cessation of growth (Walker and Duffus, 1980).

The results for the ethanol yield (Figure 3.8) are similar to those for the biomass yield with a linear relationship between the initial magnesium concentration and the ethanol yield existing for only a very narrow range of magnesium with a plateau

occurring at 75μM. The maximum value obtained (0.42g Ethanol (g Glucose)$^{-1}$) indicates a conversion efficiency of 82% based on the Guy-Lusac equation.

Calculated results for the biomass and ethanol yields as a function of the amount of magnesium used are shown in Figures 3.9 and 3.10 respectively. The amount of magnesium used was calculated by subtracting the minimum concentration of magnesium reached from the initial concentration of magnesium within the medium. The graphs show a clear exponential decline in yield as a function of the initial magnesium concentration with the leveling out of the response again at approximately 650μM.

The graphs of the ethanol production rate (Figure 3.11) and the glucose uptake rate (Figure 3.12) are very similar to each other and to the growth rate. Neither curve passes through the origin and both curves achieve a plateau at approximately 625μM of medium magnesium whilst the specific quantities of glucose utilised and the quantities of ethanol produced reflect the conversion efficiency mentioned above.

The cells cultured in the lowest magnesium concentration produce ethanol but as the concentration of magnesium increased the cell concentration rises as does the rate of ethanol accumulation. Hence, is the increase in the ethanol accumulation rate accounted for by the increase in cell concentration or is there an increase in the fermentative activity? In an attempt to address this issue, the ethanol production rate has been recalculated on a per cell basis and the results are presented in Figure 3.15 below.

Figure 3.15: The influence of the initial magnesium concentration on the ethanol producion rate expressed on a dry weight basis (+) and on a per cell basis (■)

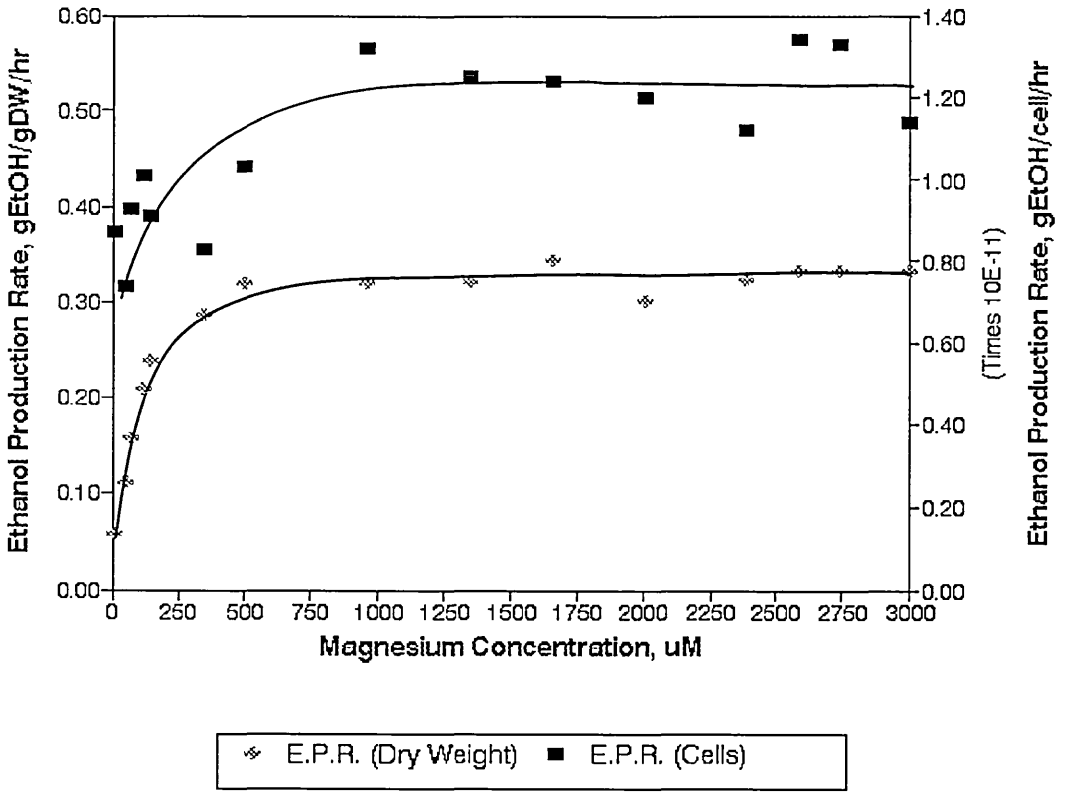
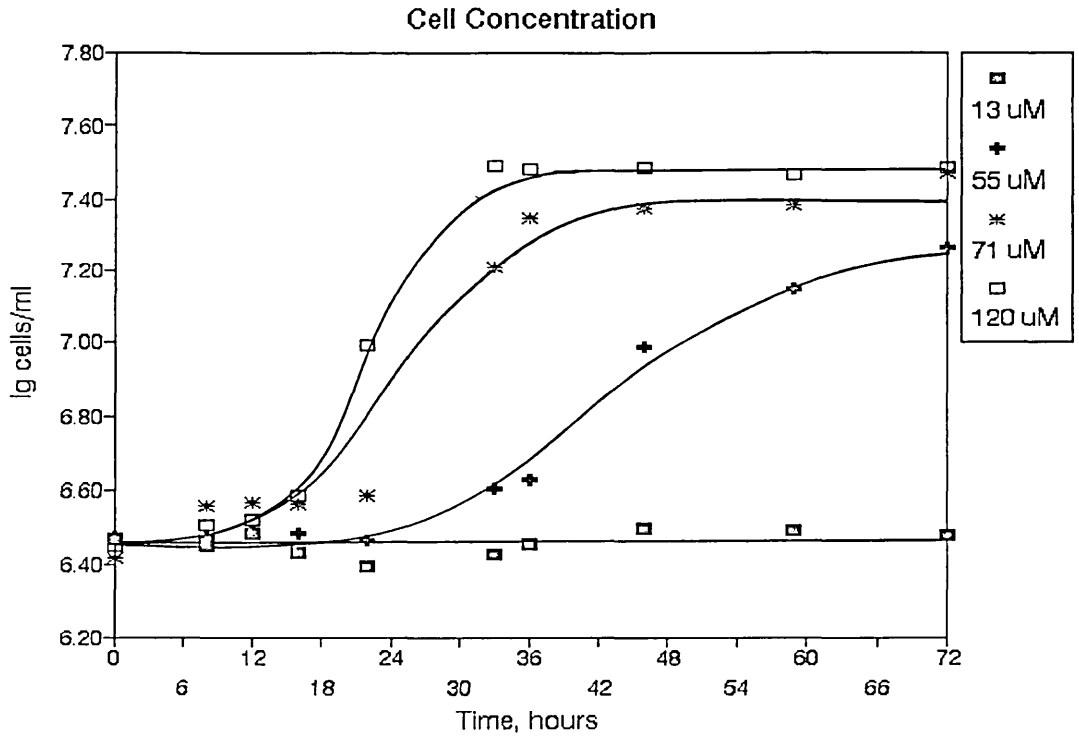


Figure 3.15 shows that the ethanol production rate on a per cell basis is broadly similar to the ethanol production rate on a dry weight basis and that there is therefore an increase in the fermentative activity of the individual cells rather than the increase in the ethanol production rate being due to a simple increase in cell numbers.

Figure 3.1: The influence of the initial medium magnesium concentration on the yeast cell concentration during batch fermentations. Refer to legend for key to symbols.



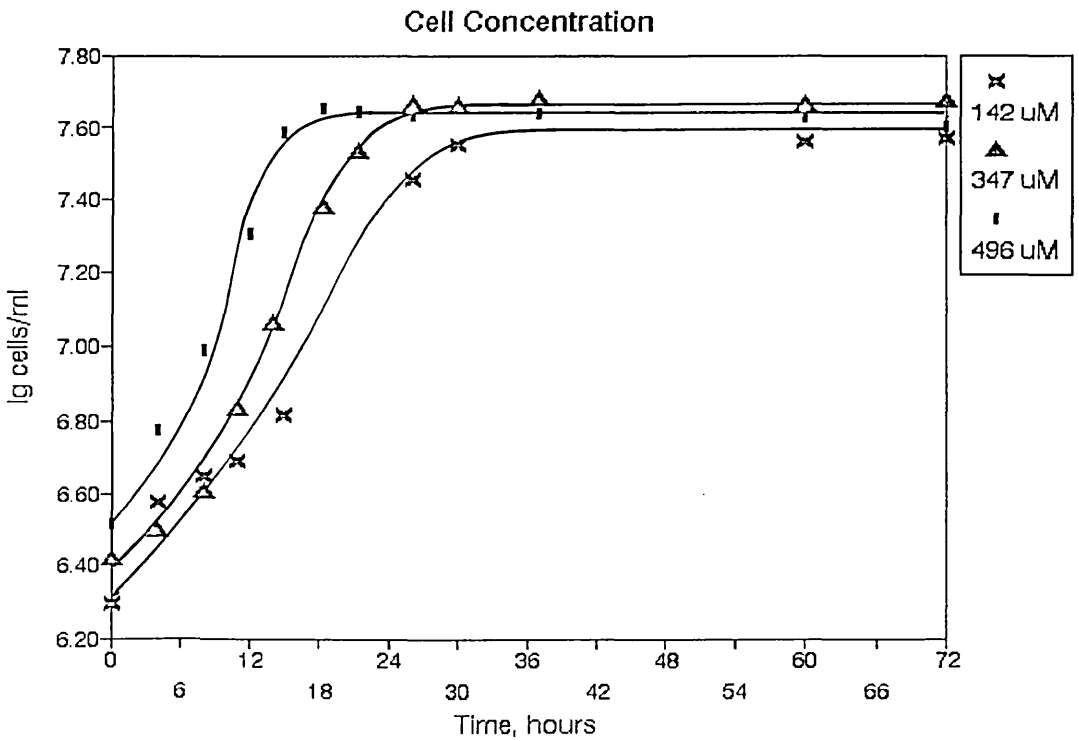
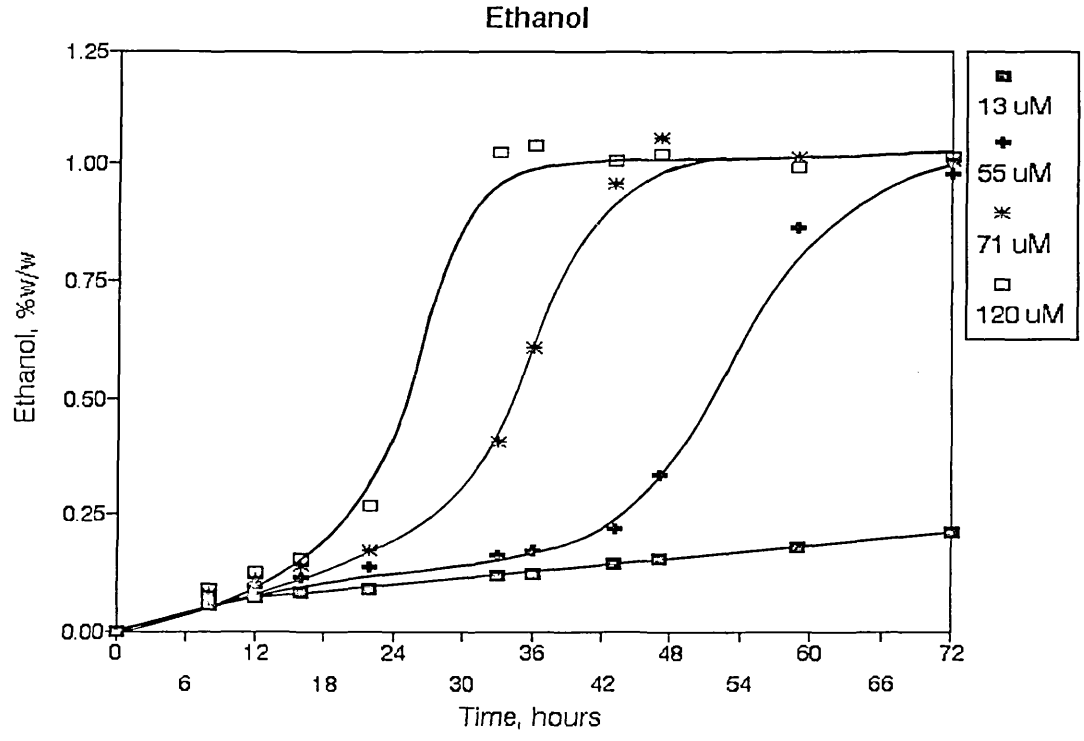


Figure 3.2: The influence of the initial medium magnesium concentration on the ethanol concentration within the medium during batch fermentations. Refer to legend for key to symbols.



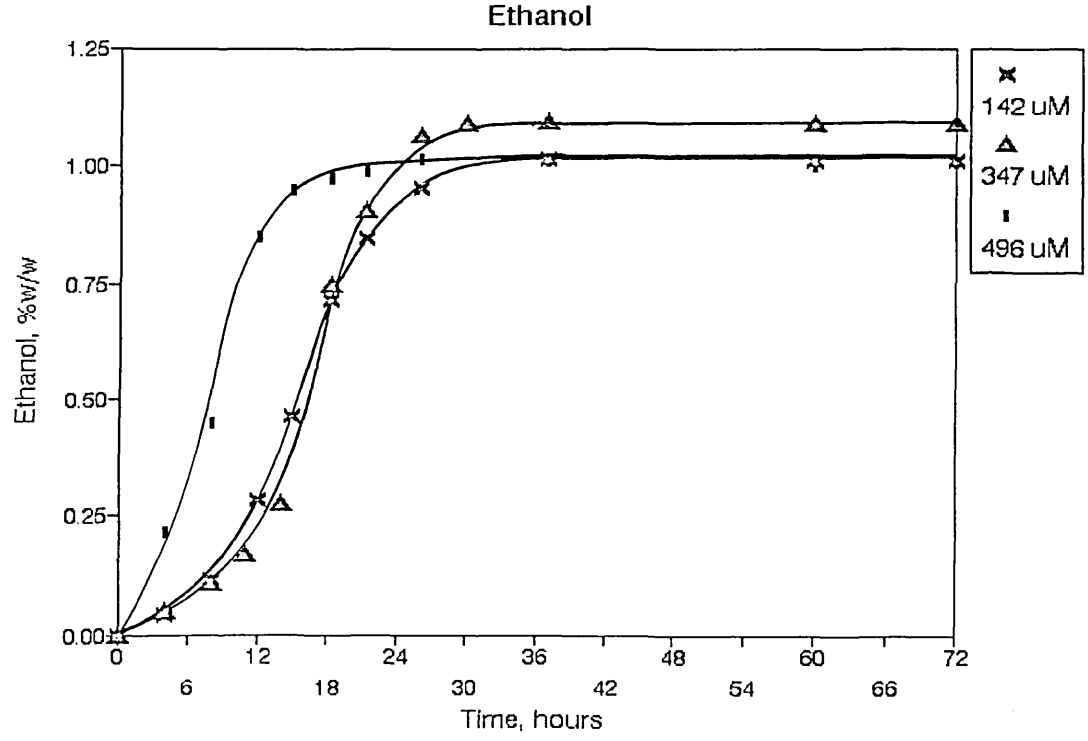
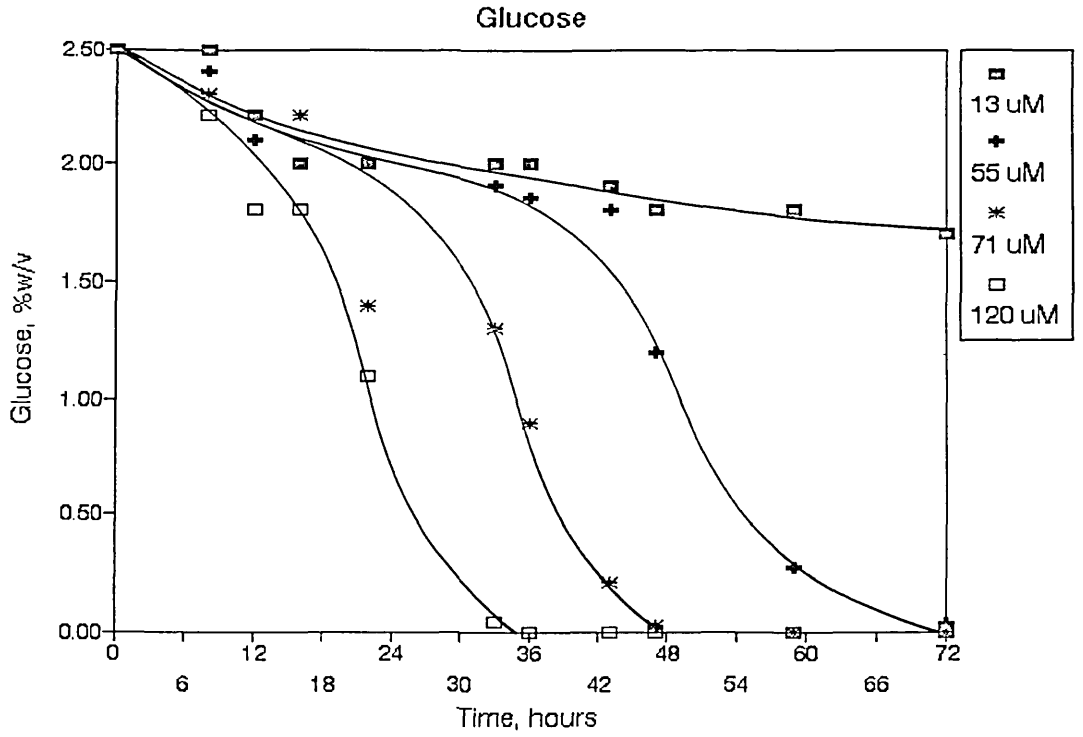


Figure 3.3: The influence of the initial medium magnesium concentration on the glucose concentration within the medium during batch fermentations. Refer to legend for key to symbols.



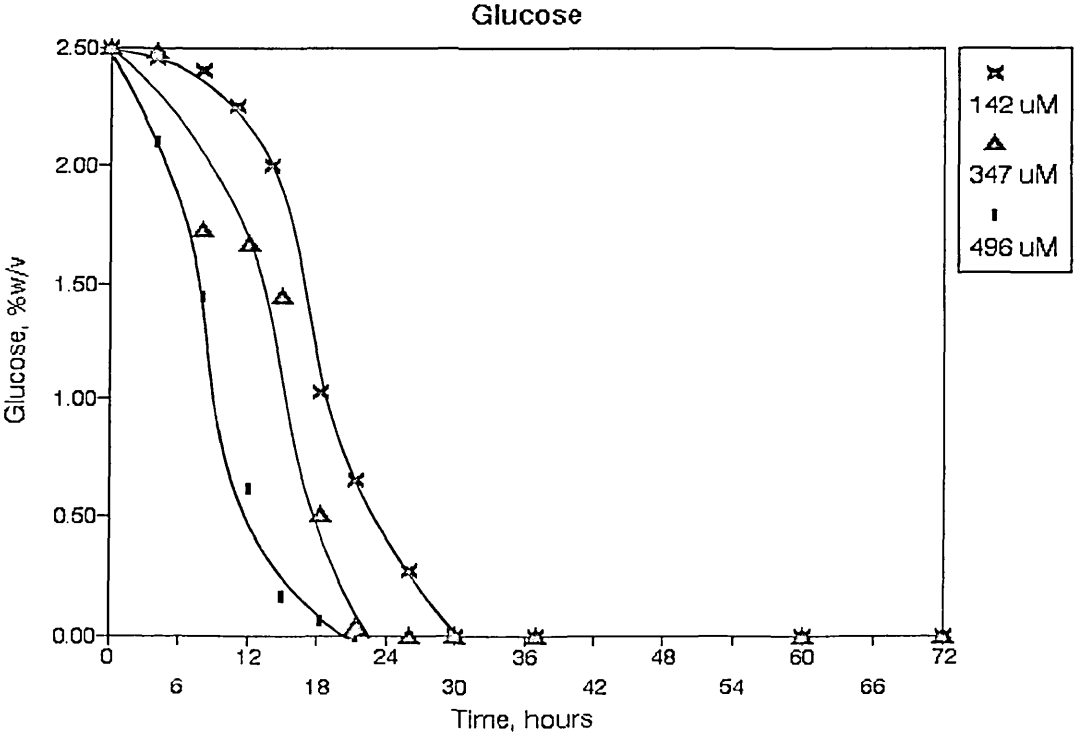
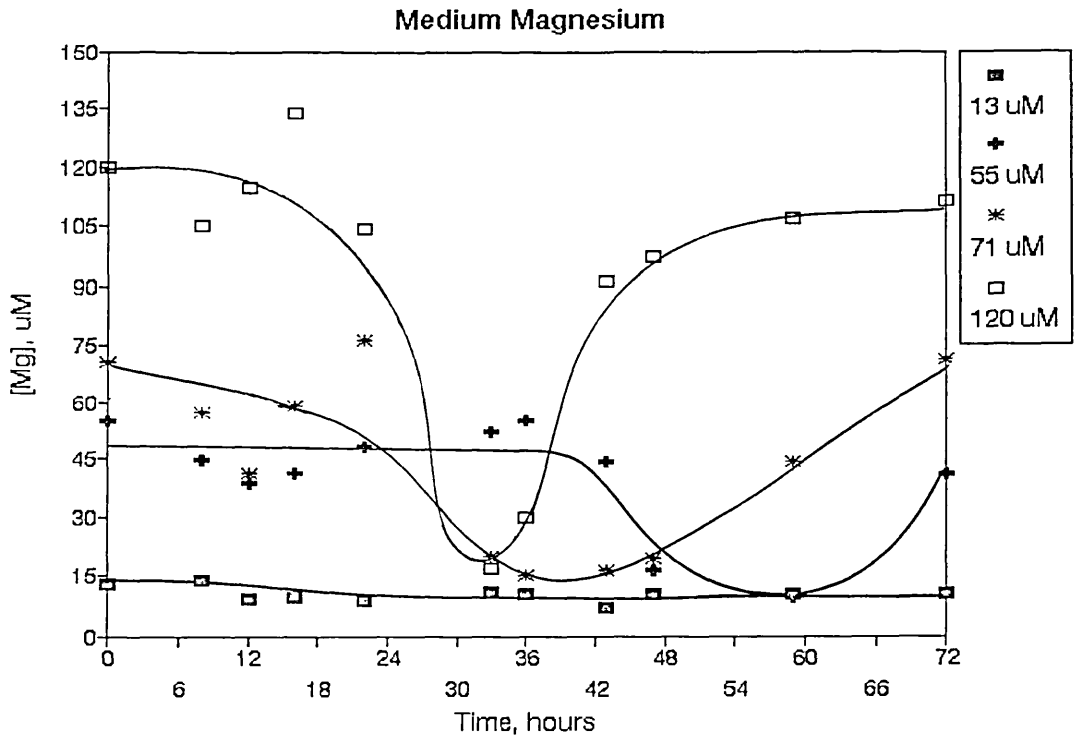


Figure 3.4: The influence of the initial magnesium concentration on the magnesium concentration within the medium during batch fermentations. Refer to legend for key to symbols.



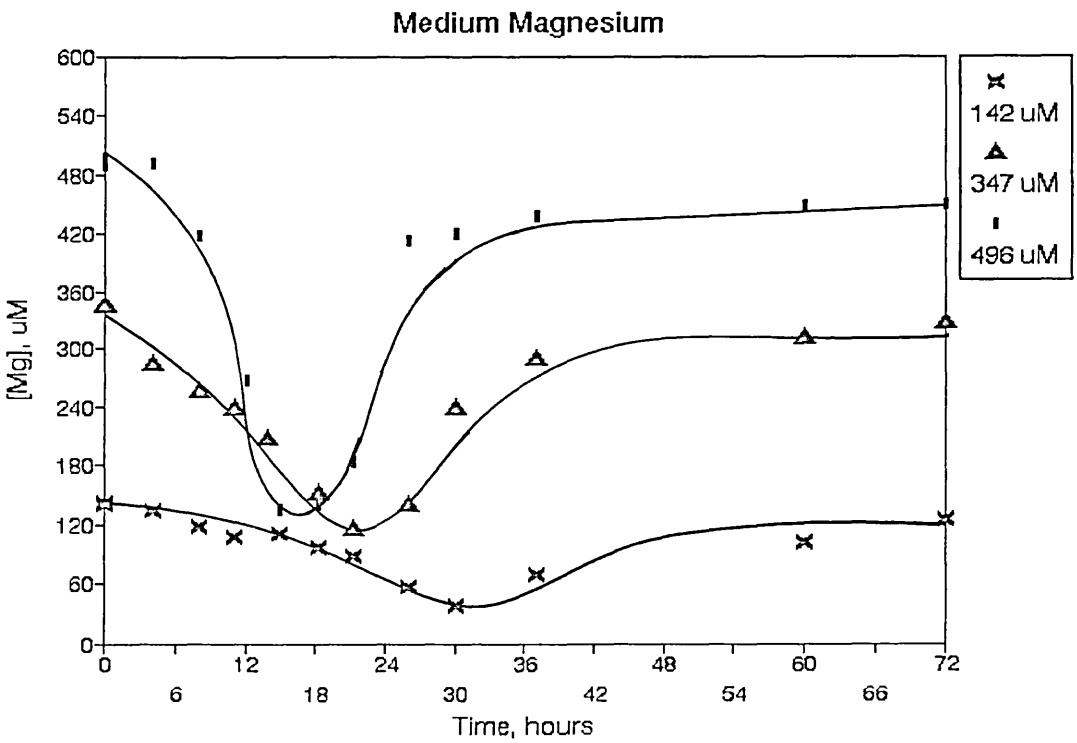


Figure 3.5a: The relationship between the yeast growth rate and the initial magnesium concentration within the medium during batch fermentations.

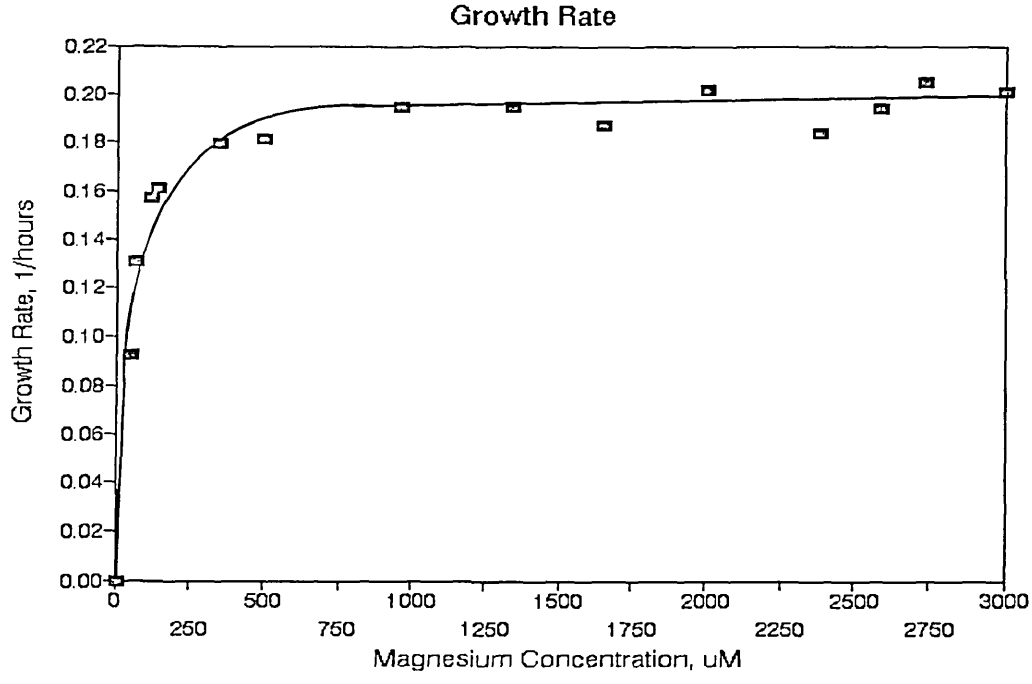


Figure 3.6: The relationship between the yeast ethanol productivity and the initial magnesium concentration within the medium during batch fermentations.

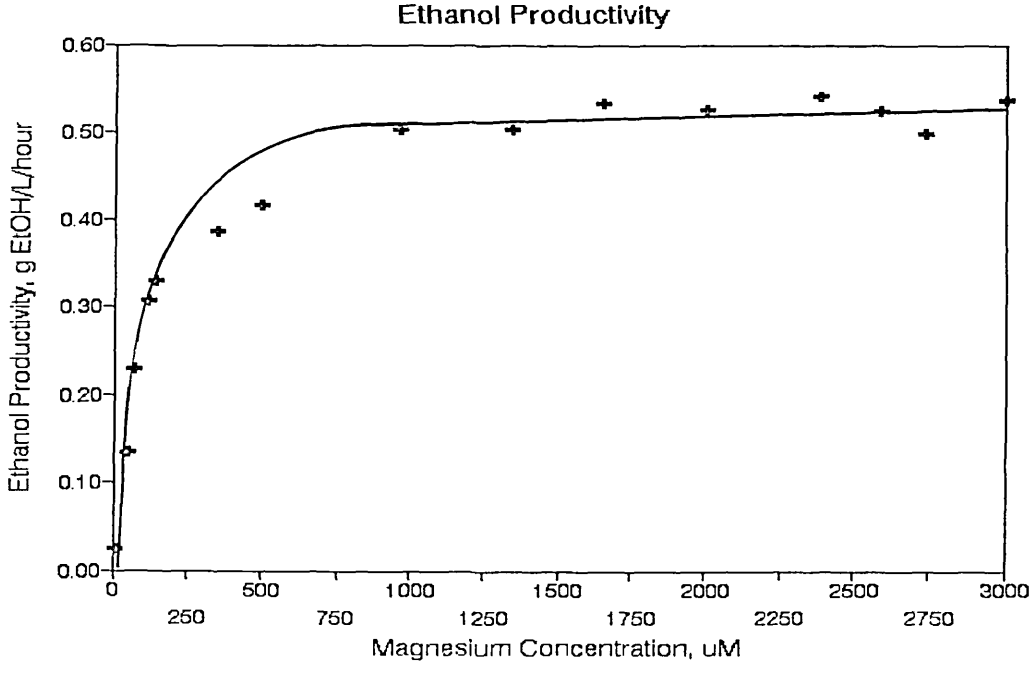


Figure 3.7: The relationship between the yeast biomass yield (glucose) and the initial magnesium concentration within the medium during batch fermentations.

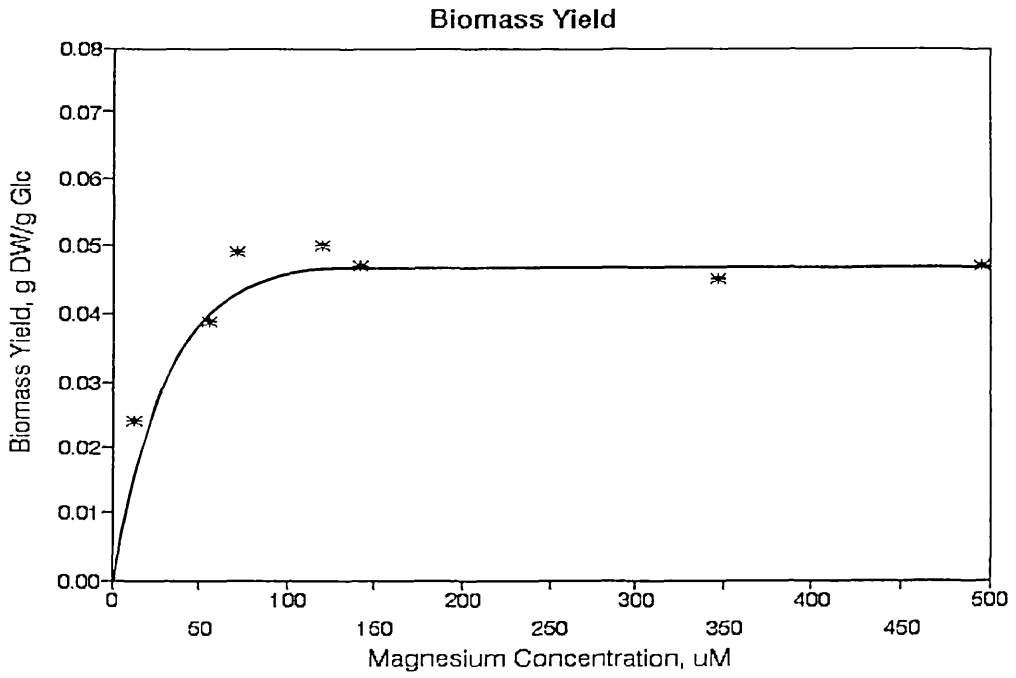


Figure 3.8: The relationship between the yeast ethanol yield (glucose) and the initial magnesium concentration within the medium during batch fermentations.

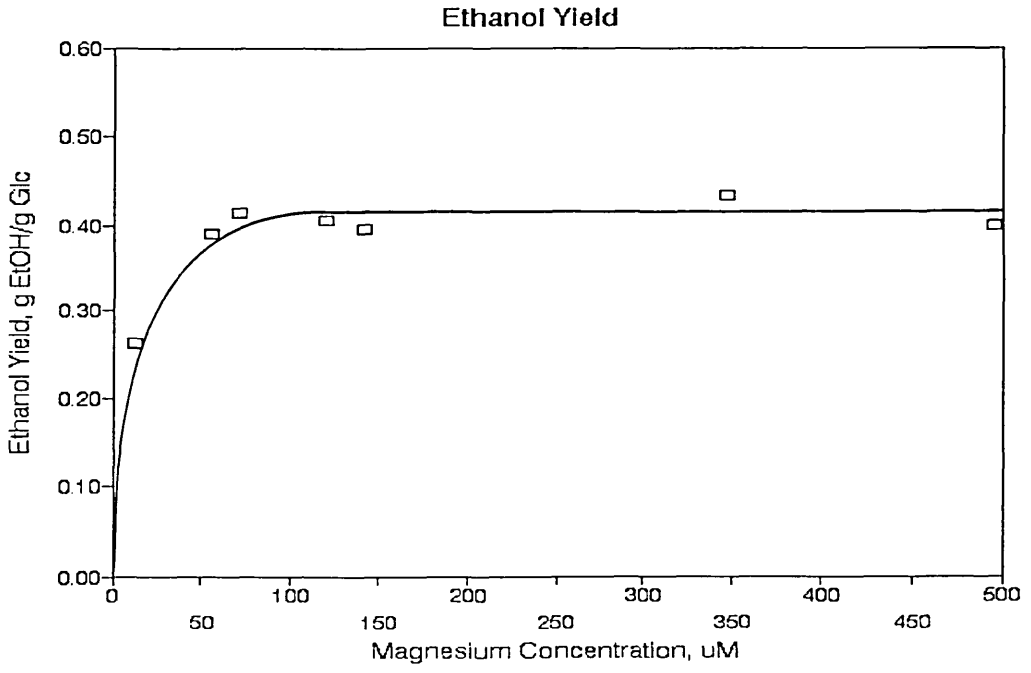


Figure 3.9: The relationship between the yeast biomass yield (magnesium) and the initial magnesium concentration within the medium during batch fermentations.

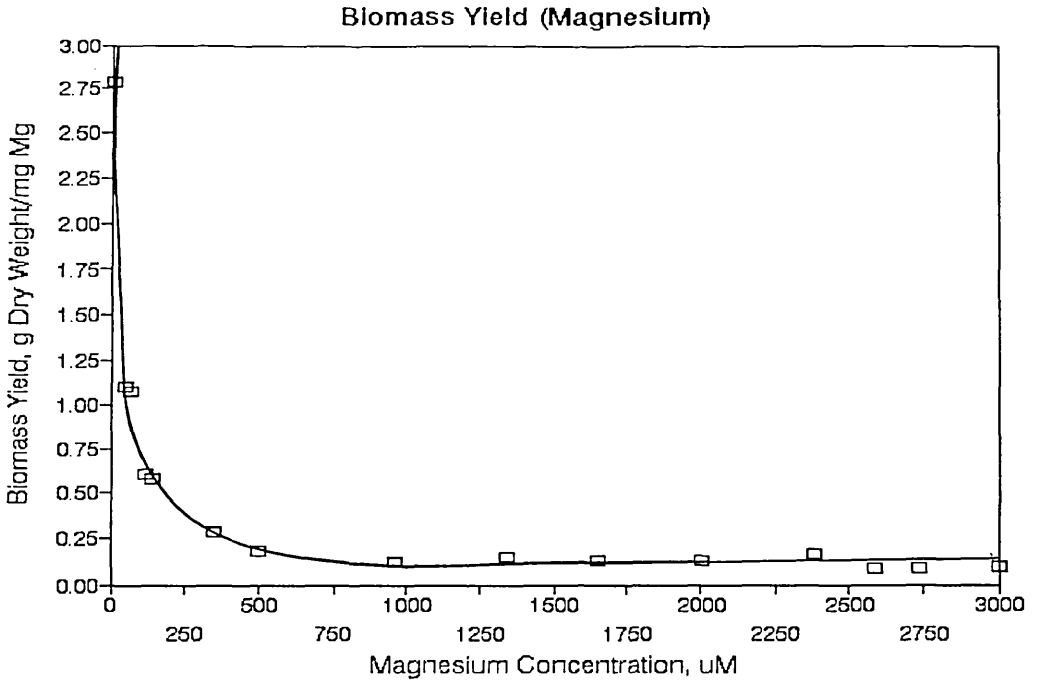


Figure 3.10: The relationship between the yeast ethanol yield (magnesium) and the initial magnesium concentration within the medium during batch fermentations.

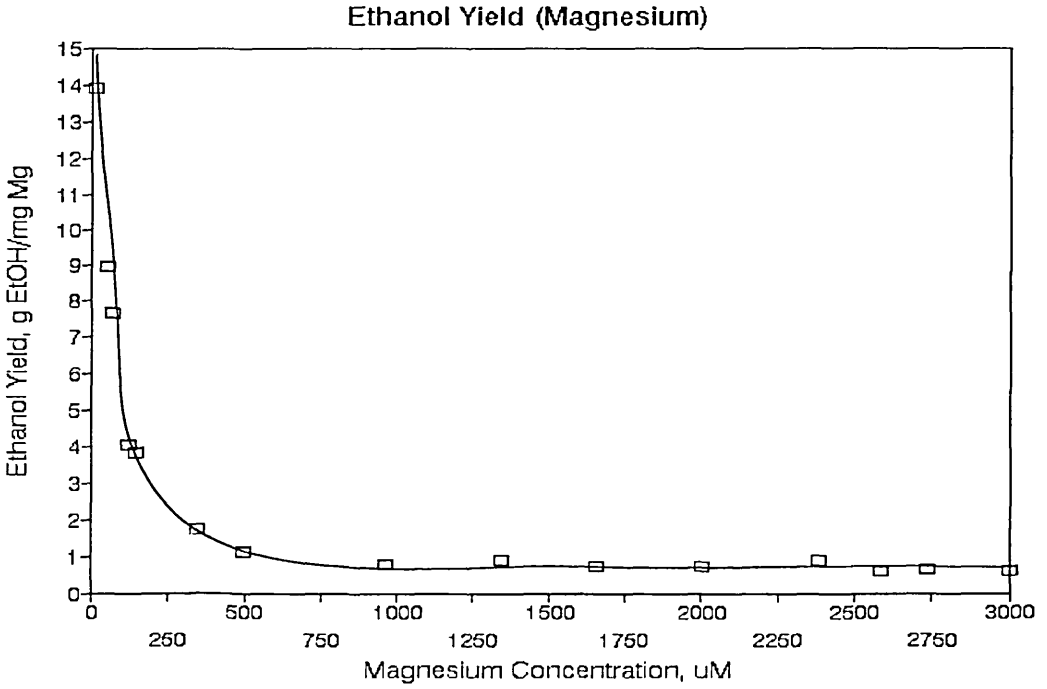


Figure 3.11: The relationship between the yeast ethanol production rate and the initial magnesium concentration within the medium during batch fermentations.

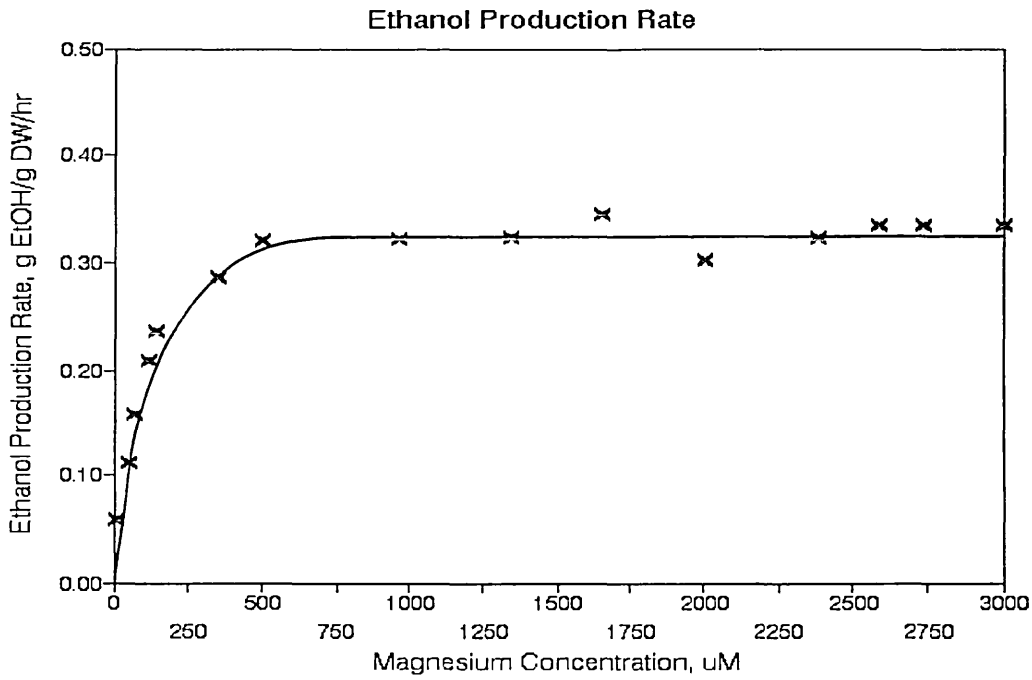
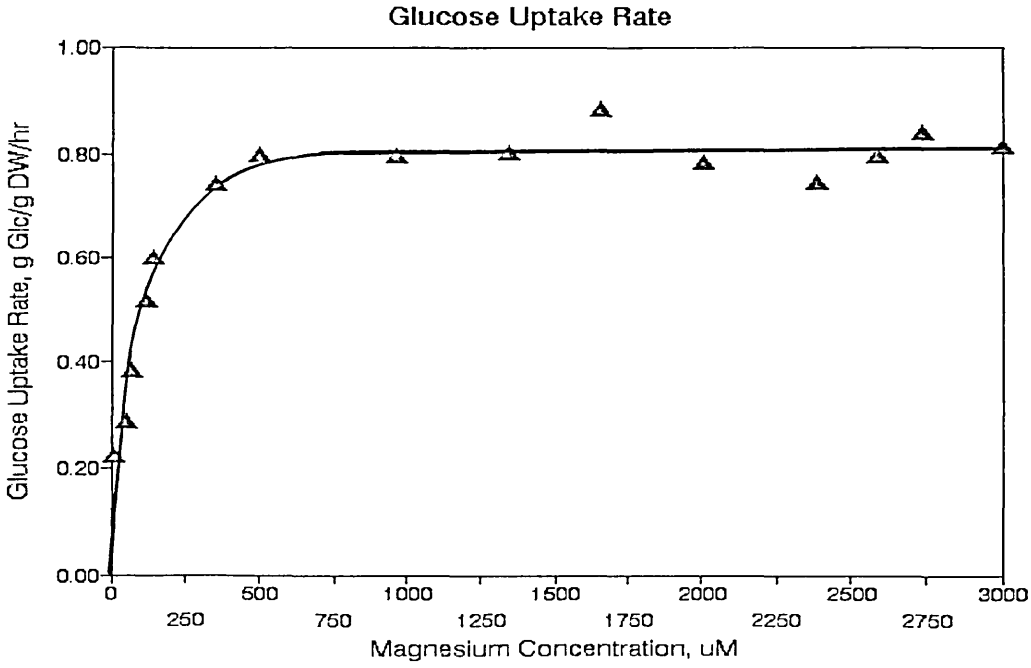


Figure 3.12: The relationship between the yeast glucose uptake rate and the initial magnesium concentration within the medium during batch fermentations.



3.1.2. The Effect of Inoculum Magnesium Levels on Batch Fermentation Parameters

3.1.2.1. Introduction

The properties of the yeast cell are ultimately determined by the genome, however, the cell can adapt to its environment either by altering the genome through spontaneous mutations or by phenotypic adaptation (Meyer *et al.*, 1985). The latter method is obviously affected by the environment and is fundamental to biotechnology with the physical and chemical environment determining the final qualities of the microbial population (Meyer *et al.*, 1985).

There is much ongoing research into the control of cell populations through the manipulation of the immediate environment as the efficient utilization of any medium is dependent on establishing the appropriate conditions to meet the cellular metabolic requirements necessary for the desired substrate-to-product conversion. This requires a detailed understanding of the pertinent metabolic pathways and the cell's requirements for key nutrients that must be supplied at the correct time, in adequate amounts and in the optimum chemical species. It has been stated that brewers have only a limited control over the yeast and the fermentation (Hudson, 1983), with control options primarily being the composition of the wort through choice of grain, mashing conditions, pitching rate and temperature control (Stewart and Russell, 1986).

However, a complementary alternative to manipulating the environment of the main production culture is to exert control 'pre-fermenter' (Quain, 1988), i.e. either by 'conditioning' the inoculum to the main fermentation medium and thus force any required physiological adaptations to occur before transfer or by anticipating the future nutrient requirements of the cell and using appropriate techniques to enhance the accumulation of the nutrients in question.

Growth of yeast cells on glucose has been shown to be influenced by the incubation of the cells in a variety of conditioning environments including different carbon sources (Bruver, Ball, & Tustanoff, 1975), different sugar concentrations (Ball, Bruver, & Tustanoff, 1975), various aeration regimes (Hauklie and Lie, 1973), altered inoculum levels (Strehaiano, Mota, & Goma, 1982), and different physiological ages

(Tustanoff and Bartley, 1964). It is known that the standard deviation found in the composition of yeasts for the major elements is +/- 70% (Jones and Greenfield, 1984) which has not been satisfactorily explained but may prove useful in developing a conditioning technique.

Barford (1985) used adapted inocula to obtain batch data comparable to continuous culture data where the adaptation involved fresh shake-flask incubations of the inoculum cells every 24hrs for a two-week period in the medium to be used in the main experiment. Orlowski and Barford (1988) showed through 'adaptation' experiments that certain kinetic characteristics of fermentation and growth of a batch culture of *S. cerevisiae* grown on sucrose or fructose altered as a result of the conditions of the inoculum and the number of generations allowed to occur within the adaptation inoculum.

Borst-Pauwels (1967) observed large differences in the uptake of phosphates according to the inoculum's incubation conditions whilst the influence of structural ions on yeast growth caused by the exposure of the plasma membrane to the environment has been 'largely overlooked' (Jones and Greenfield, 1984). Cells store a variety of phosphate species in vacuoles and as intracellular metaphosphate directly influences the batch performance of the cells then the history of the culture may be more important than current phosphate concentration in the medium (Markham and Byrne, 1968; cited by Jones and Gadd, 1990). Vacuoles also play a role in maintaining the cytoplasmic concentration of magnesium (Jones and Gadd, 1990).

Conway and Beary (1962) produced baker's yeast cells that contained high levels of magnesium and low levels of potassium and which exhibited reduced growth and fermentation rates and a significantly altered morphology as compared to the non-manipulated cells. Cells rich in magnesium but with normal potassium levels showed no significant differences to the 'normal' cells. The authors concluded that the changes seen were due to potassium deficiency and not excess magnesium, a conclusion verified when normal growth and fermentation were resumed after the addition of exogenous

potassium chloride to the high magnesium/low potassium cells. No enhancement of growth or fermentation was observed in the high magnesium/normal potassium cells.

Further examples of 'conditioning' are found in the yeast production industry where the nutritional 'priming' of the cells before drying is an established protection technique whereby yeast cultures primed to different extents with various nutritional mixes are supplied to different customers who have different expectations and requirements of the yeast population (Jensen, 1990).

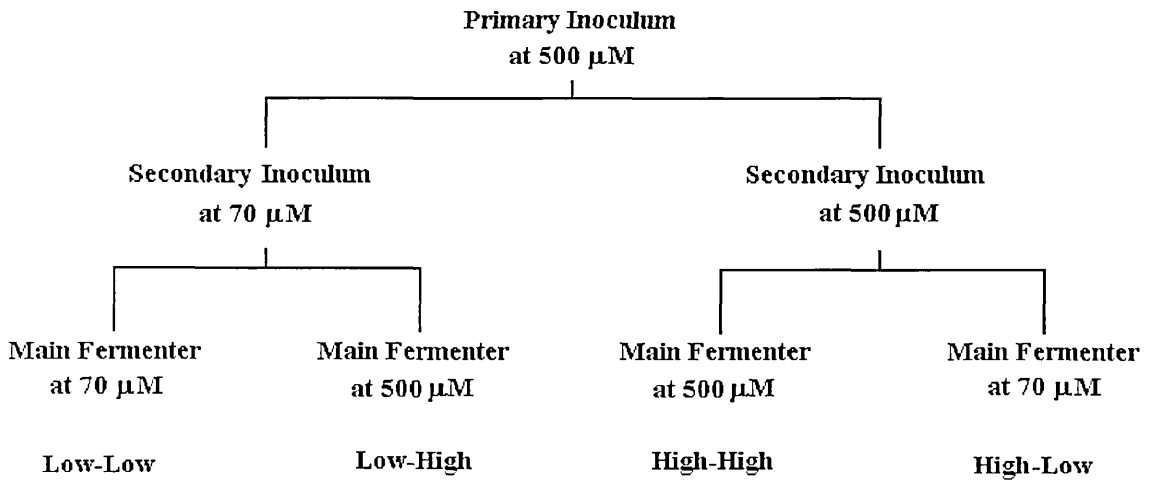
The aim of the following experiment was to observe the influence of the exogenous magnesium concentration within the inoculum medium on the fermentative capacity of the yeast population and to determine whether the ratio of inoculum magnesium concentration to main fermenting medium magnesium concentration affected the yeast's fermentative behaviour.

3.1.2.2. Method

The methodology for the fermenter set-up is detailed in section 2.1.1; the medium preparation in section 2.1.2; and the inoculum preparation in section 2.1.3. Changes to the preparation of the inoculum were as follows. The cell culture from the primary inoculum was divided in half and used to initiate two secondary cultures. The first had an initial magnesium concentration of 70µM whilst the second was at 500µM. After incubation the cells were harvested as described in section 2.1.3 although each flask's contents were divided in half and the aliquots used to inoculate the four main fermenters. The magnesium concentration of two of the main fermenters was set at 70µM whilst the remaining fermenters were set at 500µM. Hence, the cells in the first fermenter had been cultured at 70µM and were fermenting a 70µM medium (i.e. 70-70µM) whilst the cells in the second fermenter had also been cultured at 70µM but were fermenting a 500µM medium (70-500µM). The third fermenter cells had been cultured at 500µM and were fermenting a 500µM medium (500-500µM) whilst the fourth

fermenter cells had been cultured at 500μM but were fermenting a 70μM medium (500-70μM). This procedure is represented schematically in Figure 3.16 below.

Figure 3.16: Schematic representation of the "magnesium conditioning" inoculating procedure



The sampling regime is described in section 2.1.4 whilst the procedure for the analyses of samples is detailed in section 2.3.

The experiment was repeated and whilst the trend within the results of the second experiment are in general agreement with the results presented here, the level of cells within the inocula were too low to consider the experiment a strict repeat. In brief, the lowered cell concentrations seemed to accenuate the differences between the cultures' fermentation parameters although stationary phase was not reached.

3.1.2.3. Results and Discussion

The effect of the ratio of the inoculating medium magnesium concentration to the fermenting medium magnesium concentration on the cell's fermentation parameters (cell concentration, mean cell volume, ethanol, glucose, medium magnesium levels, and cellular magnesium levels) is shown in Figures 3.17, 3.18, 3.19, 3.20, 3.21, and 3.22 respectively. The effect on derived fermentation variables such as the specific growth

rate and ethanol productivity, the biomass and ethanol yields, and the ethanol production rate and glucose uptake rate are shown in Figures 3.23, 3.24, 3.25, 3.26, 3.27, and 3.28 respectively.

From Figure 3.17, slight differences between the culture are noted with most significant being that the cultures within the main fermenting medium containing 70μM do not attain the same level of cell numbers as the cultures within the 500μM medium; a result that is not in accord with the results presented earlier (section 3.1.1). Referring to Figure 3.23, it is more clearly seen that the cells within the 70μM main fermenting medium also exhibit the lowest specific growth rates although it would appear that the growth rate of the cells cultured within the inoculating medium containing 500μM magnesium is higher than that of the cells cultured at 70μM. This trend is also present within the cells fermenting the 500μM main fermenting mediums.

These differences between the cell populations according to their culture conditions appear to influence the rate of ethanol accumulation and the rate of glucose depletion within the main fermenting mediums (Figures 3.19 and 3.20 respectively). The final levels of ethanol produced are similar but the rate at which it is produced is slowest in the 70-70μM culture and fastest within the 500-500μM culture as a function of both medium volume (Ethanol Productivity: Figure 3.24) and cell dry weight (Ethanol Production Rate: Figure 3.27). The only contradiction to these general trends is seen in the rate at which glucose is removed from the medium: 500-500μM, 70-70μM, 70-500μM, and 500-70μM (Figure 3.28).

Differences are observed between the cellular ethanol productivities of the cultures (Table 3.2 below) which are again in keeping with the general trend emerging.

Table 3.2: The influence of the magnesium concentration within the inocula relative to the magnesium concentration within the main fermenting media on the cellular ethanol productivity and the ethanol production rate.

Inoculum Magnesium Concentration - Fermentation Magnesium Concentration (μM)	Cellular Ethanol Productivity (g EtOH per cell per hr)	Ethanol Production Rate (g EtOH per g dry weight per hr)
70-70 (Low to Low)	18.2×10^{-12}	0.316
500-500 (High to High)	21.2×10^{-12}	0.333
70-500 (Low to High)	14.8×10^{-12}	0.288
500-70 (High to Low)	20.3×10^{-12}	0.281

In agreement with the results from section 3.1.1, the ethanol yield (Figure 3.27) appears uninfluenced by either the concentration of magnesium within the main fermenting medium or the inoculum culture medium. However, the biomass yield (Figure 3.25) is influenced by the experimental conditions although because this experiment was terminated sooner than the experiment within section 3.11 it is possible that the dry weight for the low-to-low culture may not have reached its final value.

Medium magnesium levels (Figure 3.21) follow the trends observed in section 3.1.1 but with only slight differences between the cultures being observable. The cellular magnesium concentrations (Figure 3.22) appear to follow the general trend whereby the cells within the main fermenting mediums of 70μM retain their magnesium much longer than the cells within the higher magnesium concentrations although no significant differences according to the inoculum magnesium levels can be discerned.

The general trend appears to be that the ratio of exogenous magnesium concentration within the main fermenting medium relative to the inoculum magnesium concentration is of importance in influencing the fermentation performance of the cell

population. A finding in keeping with the widely-accepted view that the length of the lag phase of any given culture can be reduced by matching the inoculating medium to the main growth medium. However, an excess of magnesium within the inoculum medium appears to ameliorate the detrimental effects of a low magnesium concentration within the main fermenting medium to the point that the fermentation parameters are approximately equal to those of a cell population cultured in low magnesium concentrations (70μM) but fermented at high concentrations (500μM); a finding of use if it is not possible to identically match the inoculating medium to the main growth medium.

This work indicates an exciting approach to achieving increased control over the growth and fermentative performance of yeast cultures and whilst it is appreciated that the results are by no means conclusive it is believed by this author that the results are sufficiently positive to warrant further research. Repeats of the experiments would be carried out with the intention of monitoring the magnesium uptake patterns within the inoculating medium such that the transfer time could be more accurately controlled to within one hour of the occurrence of the magnesium minima, thus "priming" the cell's enzyme systems for the fermentation of the main medium. This study focuses on magnesium for reasons explored within the introduction but obviously conditioning could be expanded to other metal ions whilst research is being conducted into yeast adaptation to maltose uptake within wort (Ernandes, Williams, Russell, & Stewart, 1993).

Figure 3.17: The influence of the relative difference between the initial exogenous magnesium concentration within the inoculum medium and the initial exogenous magnesium concentration within the main culture medium on the yeast cell concentration during batch fermentations.

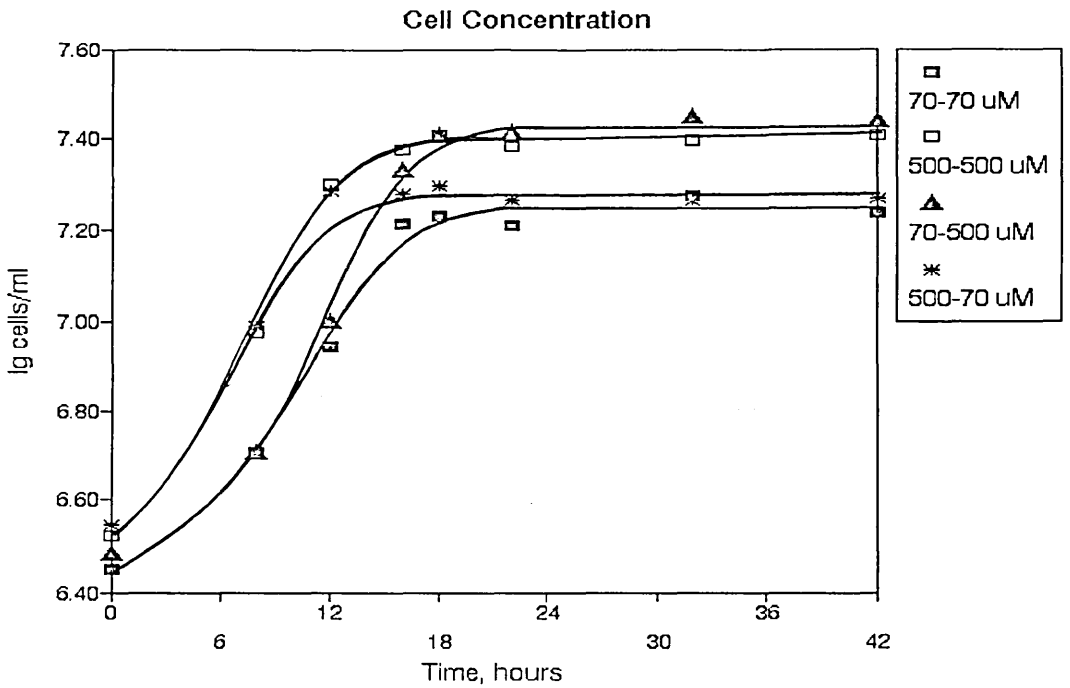


Figure 3.18: The influence of the relative difference between the initial exogenous magnesium concentration within the inoculum medium and the initial exogenous magnesium concentration within the main culture medium on the mean cell volume during batch fermentations.

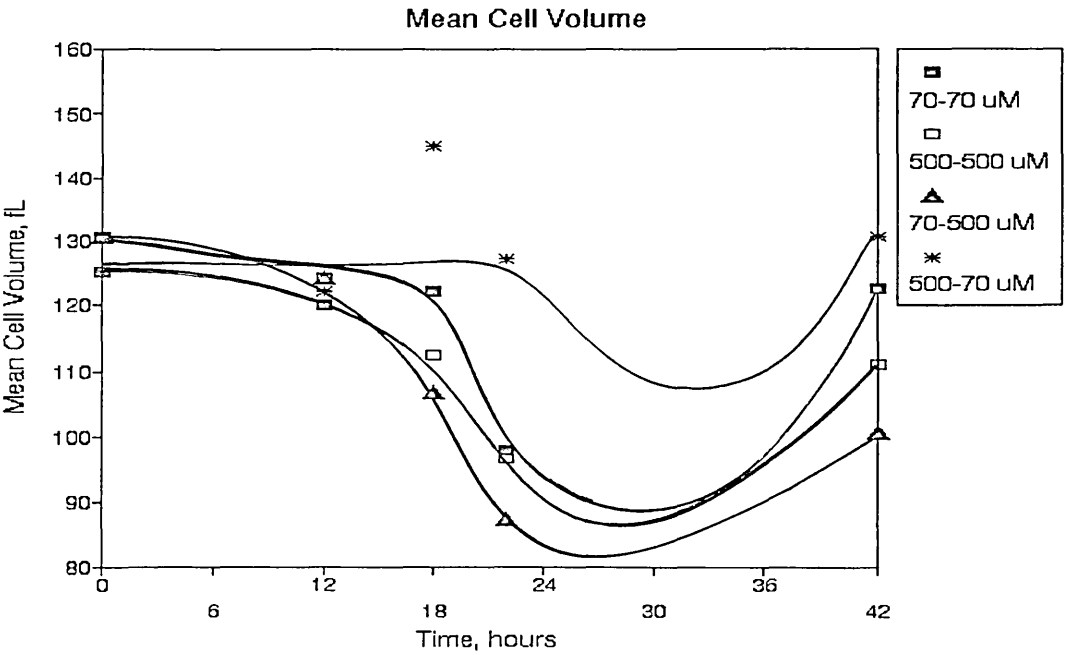


Figure 3.19: The influence of the relative difference between the initial exogenous magnesium concentration within the inoculum medium and the initial exogenous magnesium concentration within the main culture medium on the ethanol concentration during batch fermentations.

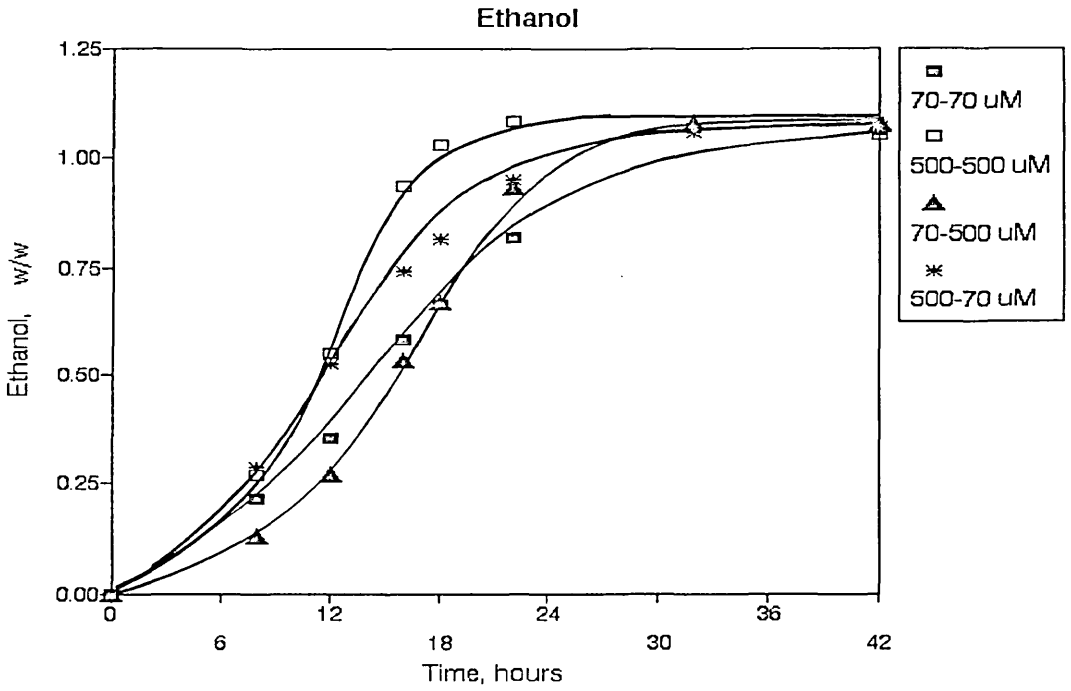


Figure 3.20: The influence of the relative difference between the initial exogenous magnesium concentration within the inoculum medium and the initial exogenous magnesium concentration within the main culture medium on the glucose concentration during batch fermentations.

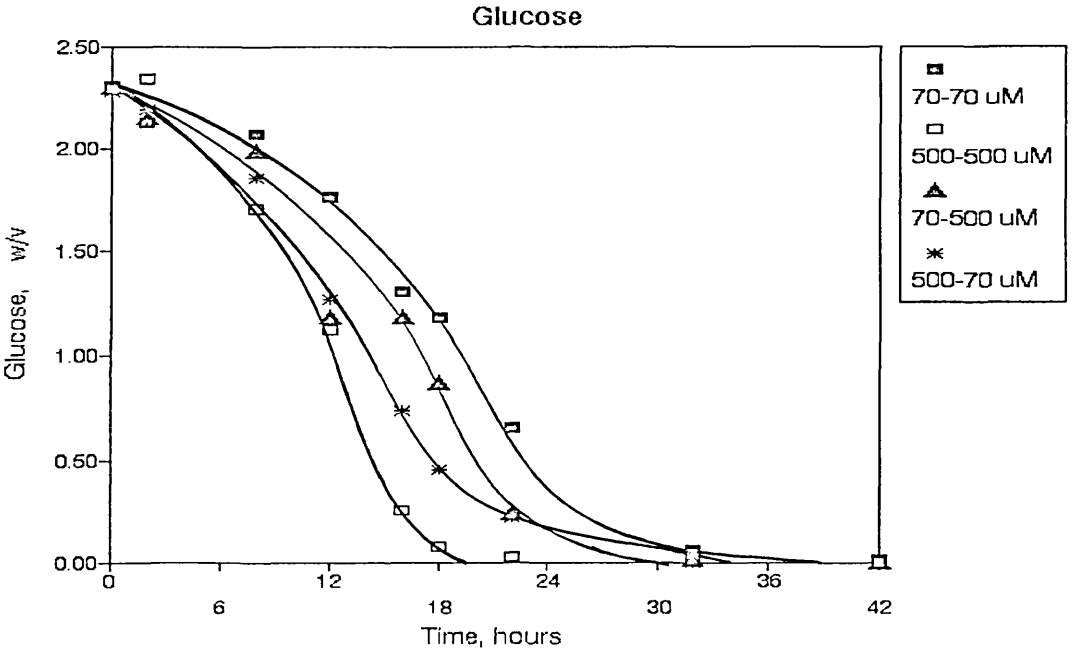


Figure 3.21: The influence of the relative difference between the initial exogenous magnesium concentration within the inoculum medium and the initial exogenous magnesium concentration within the main culture medium on the exogenous magnesium concentration during batch fermentations.

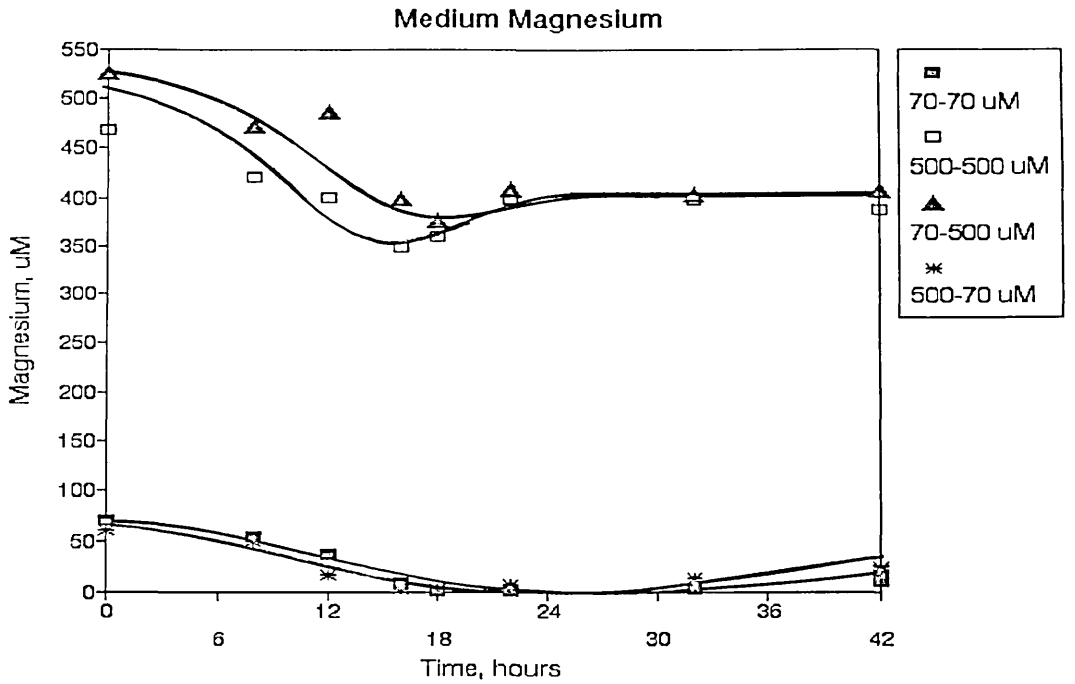


Figure 3.22: The influence of the relative difference between the initial exogenous magnesium concentration within the inoculum medium and the initial exogenous magnesium concentration within the main culture medium on the endogenous magnesium concentration during batch fermentations.

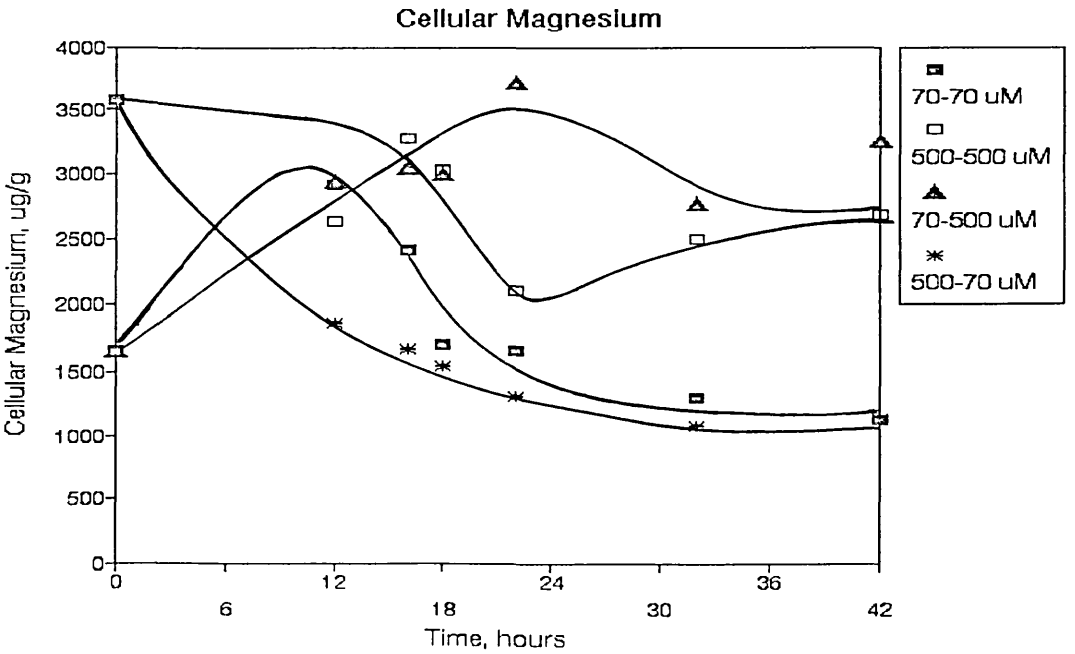


Figure 3.23: The influence of the relative difference between the initial exogenous magnesium concentration within the inoculum medium and the initial exogenous magnesium concentration within the main culture medium on the yeast growth rate during batch fermentations.

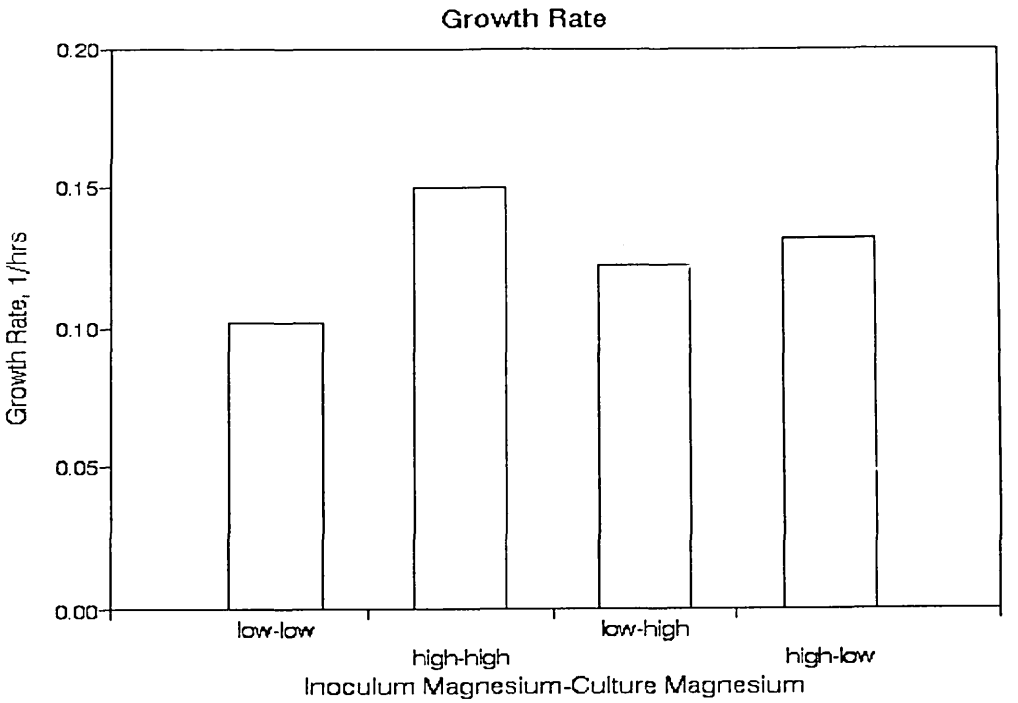


Figure 3.24: The influence of the relative difference between the initial exogenous magnesium concentration within the inoculum medium and the initial exogenous magnesium concentration within the main culture medium on the yeast ethanol productivity during batch fermentations.

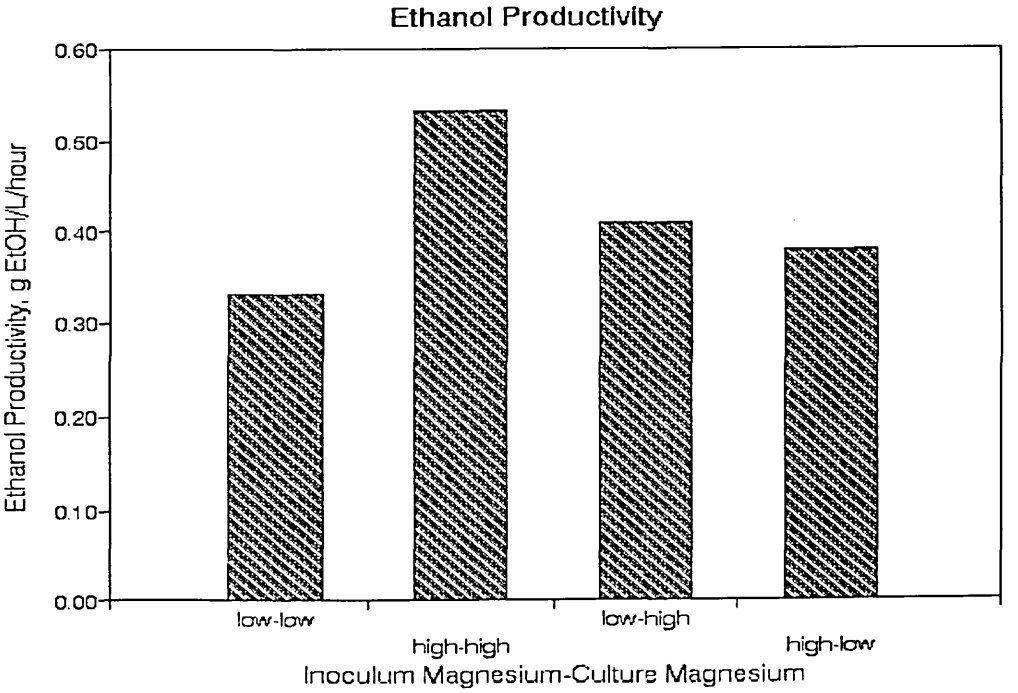


Figure 3.25: The influence of the relative difference between the initial exogenous magnesium concentration within the inoculum medium and the initial exogenous magnesium concentration within the main culture medium on the yeast biomass yield during batch fermentations.

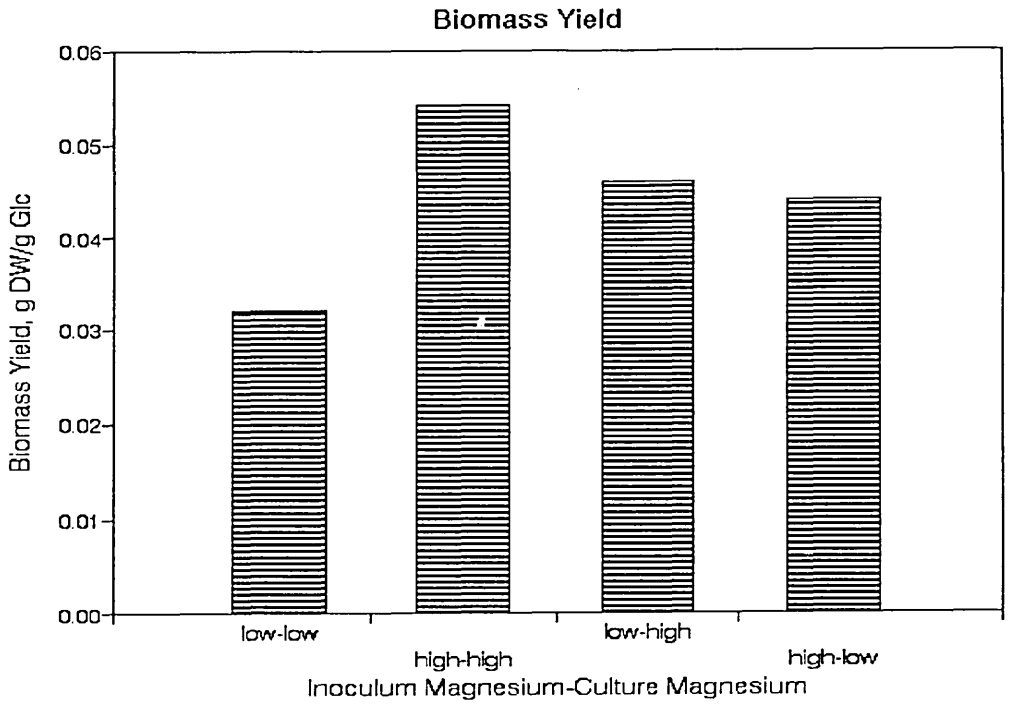


Figure 3.26: The influence of the relative difference between the initial exogenous magnesium concentration within the inoculum medium and the initial exogenous magnesium concentration within the main culture medium on the yeast ethanol yield during batch fermentations.

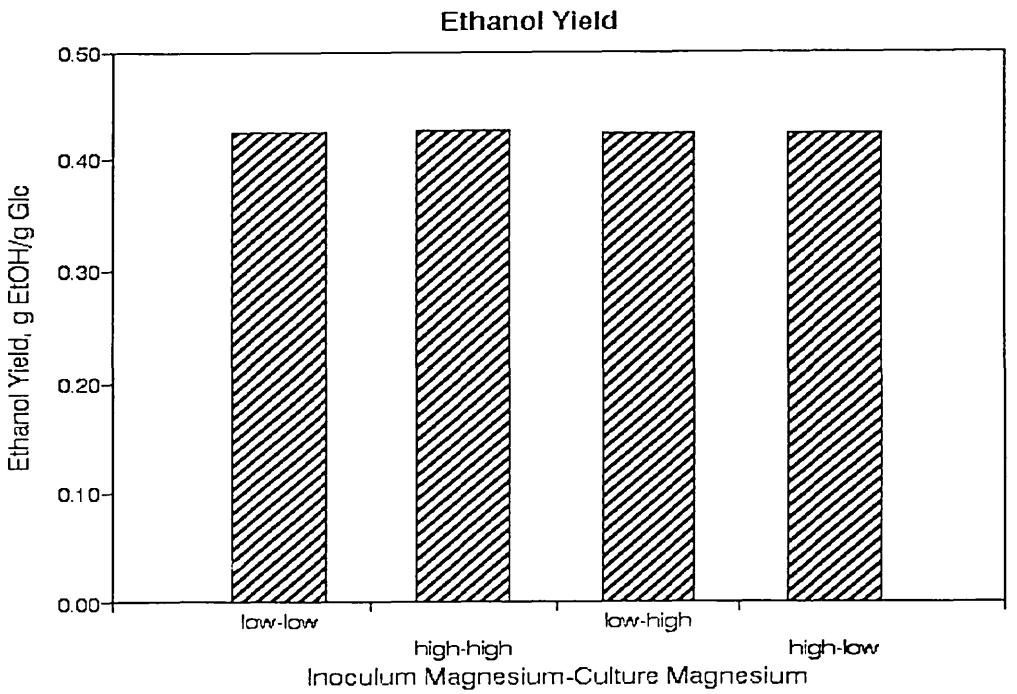


Figure 3.27: The influence of the relative difference between the initial exogenous magnesium concentration within the inoculum medium and the initial exogenous magnesium concentration within the main culture medium on the yeast ethanol production rate during batch fermentations.

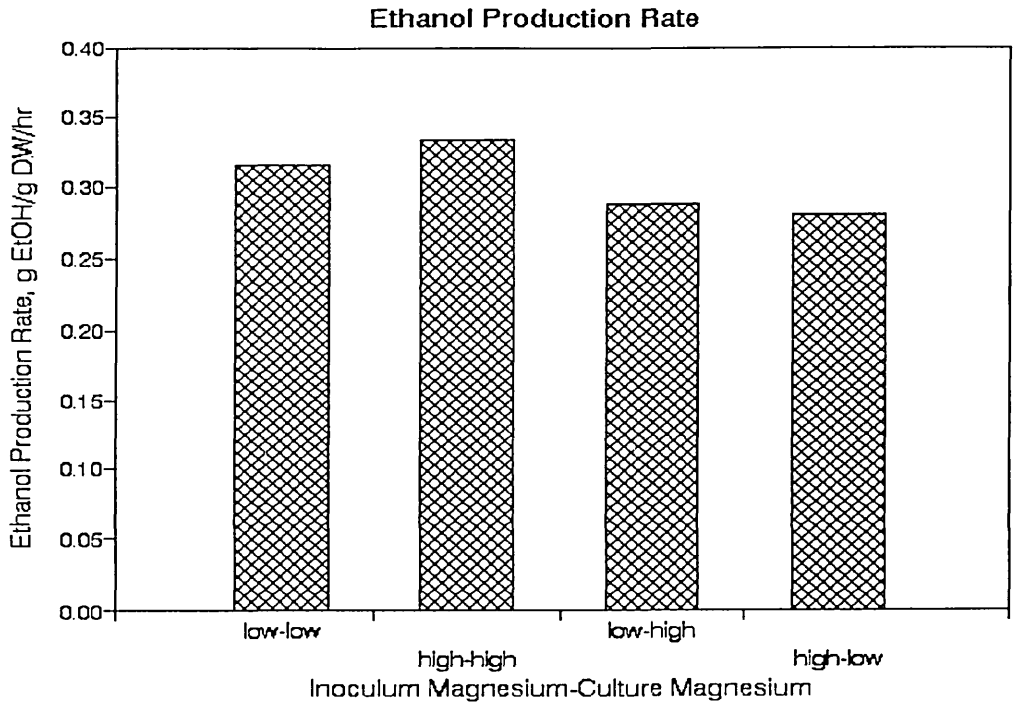
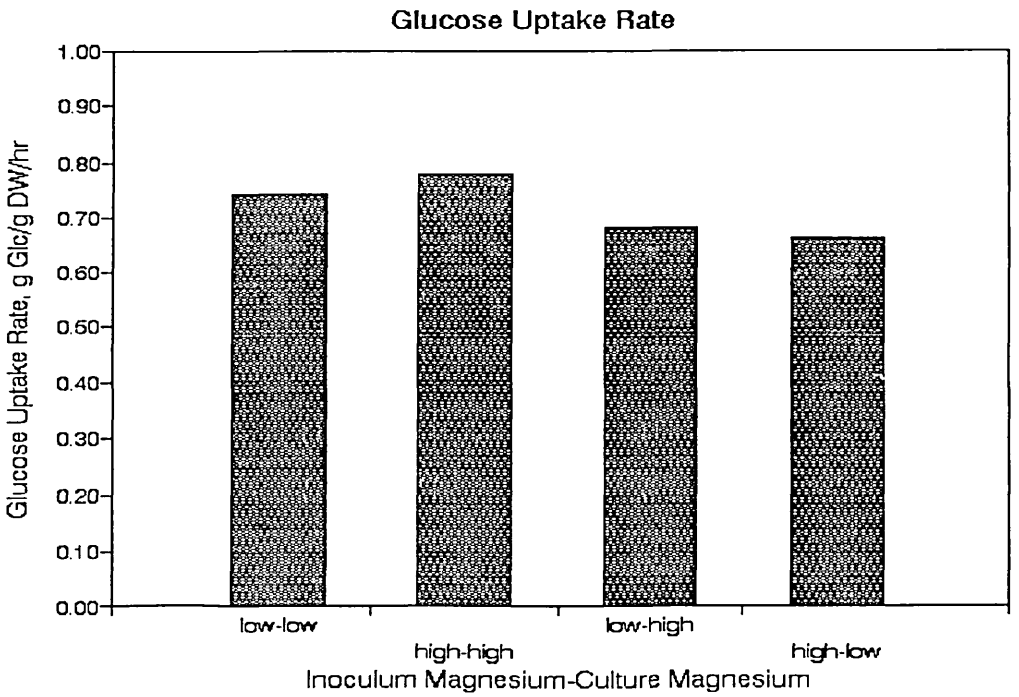


Figure 3.28: The influence of the relative difference between the initial exogenous magnesium concentration within the inoculum medium and the initial exogenous magnesium concentration within the main culture medium on the yeast glucose uptake rate during batch fermentations.



3.1.3. Magnesium Requirements within Complex Media

3.1.3.1. Introduction

The choice of substrate for the production of ethanol via fermentation is of critical economic importance as it represents the largest fraction of the production costs between 75% and 85% compared to less than 50% for petrochemical ethanol (Jones *et al.*, 1981). However, legislative demands concerning potable ethanol and the economic necessity to fully utilise resources ensures that the production of bioethanol continues. The limited substrate spectrum of ethanol-producing yeasts dictates the biomass resource to be converted (Esser & Schmidt, 1982). *S. cerevisiae* is unable to ferment pentose sugars available from wood hydrosylates; lacks depolymerizing enzymes for use on cellulose and cannot directly utilize starch or dextrins (Stewart & Russell, 1986) making substrate preparation essential. Substrates containing cellulose tend to be relatively cheap because they are usually waste products such as straw, bagasse or wood-remains although considerable costs are incurred in the chemical or physical treatments necessary to convert the cellulose into fermentable sugars (Chang, Bodoe, & Mohammed, 1981). Such transformations, at present, convert only a maximum of 60% of the cellulose (Maiorella, Blanch, & Wilke, 1984). Starch hydrolysis also requires elaborate and expensive pre-treatments (Esser & Schmidt, 1982) except within the brewing industry where classical malting techniques reduce the cost of obtaining fermentable sugars (MacLeod, 1977; cited by Esser & Schmidt, 1982). Additional expense is often incurred in the form of additives such as nitrogen, phosphate and vitamins, which are required to bring the chosen substrate up to the growth requirements of the yeast. Transport and storage costs as well as seasonal availability have all to be taken into account when calculating the economy of ethanol production from organic substrates; small plants using local materials may be more economic than large plants dependent on more widespread sources (Bu'Lock, 1979).

Plants are complex organisms, from which starting point, all the factors impinging on the processing of the crop to the end-product, such as seasonal variations, harvesting techniques, storage, handling and processing parameters, will enhance the

complexity of the by-product; commensurate with the degree of purity achieved in the main product. A prime example is seen in the sugar-cane crop, whose purification leads to refined sugar as the main product and blackstrap molasses as the 'waste' by-product.

With this close-linking of ethanol production to agricultural practices, it has been found desirable to maintain a degree of flexibility between the demands of the primary product and the production of a good quality fermentable substrate as an integrated approach aims to reduce individual production costs by increasing the total recovery of a wider range of productions (Bu'Lock, 1979).

The aim of most industrial fermentation processes, regardless of the fermenting substrate, is to maximize the yield and minimize substrate waste. Current fermentation processes are already operating at 90 to 95% of the theoretical yield and hence no significant genetic improvement is expected (Tyagi, 1984) but instead, the fermentation of high-sugar media to reduce distillation costs is a promising area of investigation although a limiting factor in the fermentation of high-gravity substrates is a loss of cell viability (Day, Anderson, & Martin, 1975; cited by Stewart, D'Amore, Panchal, & Russell, 1988).

Despite the improvements which may have occurred in some fermentable substrates, molasses media is still an extremely complex and unpredictable matrix wherein the study of direct causal relationships is limited. This is in part due to the presence of large quantities of organic and proteinaceous material possessing chelating ligands such as $RCOO^-$, RHN^-, RS^-, and ROH^-. The large quantity of various cations present are believed to exhibit a wide degree of ion-ion interactions possibly resulting in the reduced availability of essential ions although some benefit may be gained through the reduced toxicity of inhibitory ions. The assumption is made that molasses media is deficient in magnesium for optimal fermentation and that the direct addition of magnesium may compensate for any deficiency experienced by the cell by saturating the buffering capacity of the chelating agents.

3.1.3.2. Methods

The primary consideration in establishing the methodology was that the laboratory-based fermentations must closely approximate current industrial molasses fermentations. This was achieved by monitoring B. P. Ltd's production of potable ethanol from molasses at their Hull Works (Chemical Division) for a total period of six months.

Fermenter Set-up

The fermenters were set-up as described for batch fermentations within a minimal medium: section 2.1.1.

Molasses Preparation

The procedure for the preparation of a molasses medium was as for the minimal medium (section 2.1.2) with the exception of the actual formulae used and hence the actual constituents used. The use of a 5-litre, volumetric flask still permitted the accurate production of sufficient molasses medium not only for the requirements of the main fermenters but also the pre-inoculation verification samples. However, unlike the minimal medium, this methodology could not be used to supply the medium for the inoculum, as a lower molasses concentration was required to avoid the Crabtree Effect; also, a phosphate addition to the inoculating medium was required to enhance cell growth. The inoculating medium was therefore made up separately in a 1-litre volumetric flask.

The formula for the molasses medium was as used by B.P. Ltd at their Hull Works for the production of potable ethanol, and was deduced by averaging the pertinent data from the logs kept by the plant operators. The logs used covered the procedures for all fermentations carried out by the operators for the preceding year; these procedures being of a fixed nature around which normal operating deviations occurred. Details of the average medium constitution for the main fermenting medium

are specified in Table 3.3, while details for the inoculating medium are given in Table 3.4.

Table 3.3: Procedure for the preparation of a molasses medium

Constituent (Formulae)	Quantity (Grams constituent added to 5L volumetric)	Medium Levels (Resultant concentration of constituents)
Molasses	1385 g	277 g/L (Specific Gravity ~ 1.090)
Nitrogen $(NH_4)_2SO_4$	2.15 g	0.43 g/L
Phosphate $(NH_4)_2HPO_4$	None added at this stage	Background Levels
Magnesium $MgSO_4.7H_2O$	Experiment dependent	Background Levels + Additions
Silicone Anti-Foam	4.0 ml	0.8 ml/L
conc. Sulphuric Acid	3.85 ml	0.77 ml/L (pH ~ 4.85)

Sterilization of the inoculating medium was carried out at the Hull Works by sparging with steam for 6 hours through the air-supply pipes and then cooling by way of the circulating water-pipes. The closest approximation to this within a laboratory context was to heat the medium *in situ* using the external water jackets. However this proved very difficult to sustain and normal autoclaving was opted for instead. No sterilization of the main fermenting medium was carried out either at the Hull Works or within these laboratory fermentations.

Table 3.4: Procedure for the preparation of molasses inoculating medium

Constituent (Formulae)	Quantity (Grams constituent added to 1L volumetric)	Medium Levels (Resultant concentration of constituents)
Molasses	134 g	134 g/L (Specific Gravity ~ 1.040)
Nitrogen $(NH_4)_2SO_4$	2.12 g	2.12 g/L
Phosphate $(NH_4)_2HPO_4$	0.28 g	0.28 g/L
Magnesium $MgSO_4.7H_2O$	None Added	Background Levels
Silicone Anti-Foam	0.8 ml	0.8 ml/L
conc. Sulphuric Acid	0.25 ml	(pH ~ 4.98)

Inoculum

One gram of compressed wet yeast was aseptically removed from the centre of a 1Kg block and transferred directly to the fermenter containing the inoculum medium. This medium was cultured under maximum aeration for 10 hours before sufficient volume was removed to inoculate the three fermenters containing the main fermenting medium. Inoculation volume was at 4.4%v/v with both inoculating ratios being equivalent to B.P.'s procedure.

Sampling Regime

Samples were obtained following the methodology described for the batch fermentations of minimal media (section 2.1.4).

3.1.3.3. Results and Discussion

Magnesium Supplementations

The delineation of the magnesium requirements of *S. cerevisiae* within a minimal medium, the observed effect of the magnesium on the yeasts' fermentative performance, and the known presence of chelating agents within a molasses medium led to the hypothesis that industrial fermentations of molasses may be limiting for magnesium. Hence, direct supplementation of the molasses media with magnesium sulphate ($MgSO_4.7H_2O$) was carried out in an attempt to improve the ability of the yeast to ferment the complex media.

It was found that the addition of more than 40,000μM magnesium to a Brazilian molasses medium increased the rate of ethanol production relative to a non-supplemented Brazilian molasses medium without actually influencing the total amount of ethanol produced although a depression of the final cell concentration and the specific growth rate was observed. Increasing the addition level to 2,500 x 10^3μM did not enhance the effect.

The experiments were repeated using $MgCl_2.2H_2O$ within a Brazilian molasses medium and the enhanced rate of ethanol formation was again observed indicating that it was the magnesium that was responsible and not the anion.

However, it was found that the addition of $MgSO_4.7H_2O$ to a Javan molasses medium had no discernible effect on any fermentation parameter relative to a non-supplemented Javan molasses medium despite addition levels in excess of 250,000μM magnesium.

A summary of these results are presented in Table 3.5 below.

Table 3.5: The influence of magnesium-supplementation on the growth rate and ethanol productivities of yeast cultures grown in molasses media

Molasses	Magnesium ($MgSO_4.7H_2O$)	Ethanol Productivity (gEtOH/L/Hour)	Growth Rate (1/hours)
Brazilian	Control	0.77	0.059
	40,000μM	1.02	0.047
	2,500 x 10^3μM	1.03	0.046
Javan	Control	0.72	0.074
	250,000μM	0.71	0.074

Magnesium-supplementations were carried out on the inoculating medium with no discernible differences materialising.

The magnesium-supplementation of certain molasses types appears to give a rate effect that may be related to the levels of sequestering agents present and/or the magnesium levels already present; i.e. the yeast cells may be having to "compete" with the sequestrants for the magnesium. This may be represented: Sequestering Ligand-Mg <==> Sequestering Ligand + Mg <==> Mg-Cell.

Purification of Molasses Media

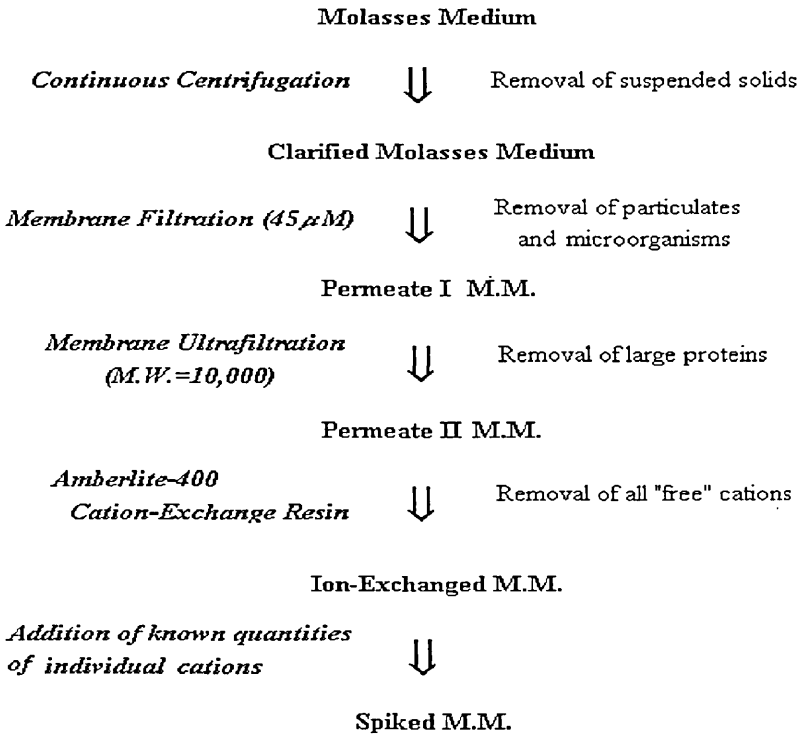
The differences in growth and fermentation parameters exhibited by the cultures within different molasses mediums is of prime interest to industrialists and it was felt that due to the complex nature of the substrate no distinct causal relationship could be directly inferred to the magnesium-supplementation experiments.

From these reservations, the idea was developed that the purification of the molasses medium in terms of the sequential removal of particulates, colloids, suspended solids and ionic fragments might be a way of establishing causal relationships. Hence, the sequential fractionation of the molasses medium followed by fermentation of the various fractions could be used as a means to explore the different limitations existing

within the molasses; whether they be direct ionic limitations or indirect sequestration effects.

The fractionation process developed was a mixture of physical methods and chemical methods and is presented in Figure 3.29 below.

Figure 3.29: Schematic diagram of the fractionation process applied to a molasses medium



The fractionation process was monitored using the coulter counter and it was found that a definite sequential removal of solids and particulates occurred. No effect on the fermentable sugars present within the original medium was observed until the medium was ion-exchanged whereupon the sucrose levels decreased significantly. However, the quantities of glucose and fructose present increased proportionately and it is believed that the protons released from the resin were responsible for the hydrolysis. The levels of sodium, potassium, and calcium were altered by the fractionation process

as detailed in Table 3.6 below. The three cations were analysed using flame photometry: a methodology that does not detect magnesium and unfortunately, no AAS was available for its detection.

Table 3.6: The effect of the fractionation process on the levels of total sodium, potassium, and calcium within the molasses medium

Fractionation Step	Sodium (ppm)	Potassium (ppm)	Calcium (ppm)	Magnesium (ppm)
Molasses Medium	103	5900	2200	953
Centrifuged Medium	95 (removal of 8%)	5891 (removal of 0.0%)	1928 (removal of 12%)	?
Permeate I	86 (removal of 9%)	5676 (removal of 4%)	1821 (removal of 6%)	?
Permeate II	59 (removal of 31%)	4498 (removal of 21%)	1499 (removal of 18%)	?
Ion-Exchanged Medium	3024 (gain of 5000%)	859 (removal of 81%)	88 (removal of 94%)	?

The various fractions of the molasses medium seem to be not so different with the processes merely removing the suspended solids. These suspended solids appear to account for only a quarter of the total ions with the remaining three-quarters of the ions being contained in the fraction separated out by the ion-exchange column. The trace levels of ions remaining in the ion-exchange effluent are presumably those ions which are bound to substances with stronger binding constants than the resin. Thus, extra layers of subtlety are required to partition the ion-exchange step; one idea being to use resins with increasing binding constants.

The various fractions were then fermented to determine whether the fractionation processes had any effect on the normal growth and fermentation of the yeast culture. A summary of the results is presented in Table 3.7 below.

Table 3.7: The effect of the fractionation process on the growth rate and ethanol productivities of yeast cultures grown within the resultant molasses fractions

Fractionation Step	Ethanol Productivity (gEtOH/L/Hour)	Growth Rate (1/hours)
Molasses Medium	1.23	0.111
Centrifuged Medium	1.27	0.115
Permeate I	1.18	0.108
Permeate II	1.09	0.105
Ion-Exchanged Medium	0.82	0.041

A slight improvement in yeast performance is observed when the molasses medium is centrifuged but thereafter performance drops as the medium is further fractionated with the most significant loss in performance occurring after the deionization step. The changes in performance broadly correlate with the changes in the total cation levels as detailed in Table 3.3 although again, no direct causal relationship has been demonstrated. The next logical step for this work is to add back or "spike" the ion-exchanged medium with specific cations and ascertain which cation has the greatest effect on yeast fermentation parameters.

This work was planned (refer to Figure 3.29 above) but was not possible within the timescale available. A further extension to this work would be to ascertain whether the magnesium uptake patterns are similar to those identified within minimal medium with the intention of optimizing the timing of the magnesium supplementations.

The results from the conditioning experiment (section 3.1.2) also indicate that it may be possible to prime the cells within the inoculating medium to ferment the main medium with greater efficiency.

3.2. Continuous Fermentations

3.2.1. Overview

Studies on the growth and metabolism of micro-organisms frequently use closed systems of fermentation (Bull, 1974). However, the data from such systems tends to be empirical and relative (Dawson, 1985) as the environmental conditions are constantly being altered by the cell's metabolic activities, with the cell continually adjusting its metabolism to the changing conditions (McDonald and Tsai, 1989). These transient conditions result in a 'history' of growth for the cell population (Bull, 1974), inadequate reproducibility (Dawson, 1985) and difficulty in assessing causal relationships (McDonald and Tsai, 1989) between growth rates and nutrient parameters (Dawson, 1985). Open systems circumvent these problems by maintaining all the conditions for growth at constant levels, ensuring that the cells are kept biochemically and physiologically constant for an indefinite time, i.e. without a 'history' (Bull, 1974). This gives controllable and reproducible results and permits the systematic study of the effects of individual environmental parameters on any organism property (van Dijken *et al.*, 1990; Bull, 1974).

Comprehensive reviews of the principles and applications of continuous culture are available in the literature (Dawson, 1985; Pirt, 1975; Bull, 1974; Herbert, 1964; James, 1961) and therefore only a summary of the main principles is presented here.

Monod's principle that the study of growth is basic to the discipline of microbiology (Monod, 1949; cited by Dawson, 1985) was the starting point for the development of the theory of continuous culture (Dawson, 1985). The Monod model relates the growth of a micro-organism in a simple defined medium, under constant environmental conditions, to the concentration of a limiting nutrient, provided all other growth requirements are present in excess (Monod, 1950; cited by Dawson, 1985). The model assumes that the cells are developing in a uniform manner under conditions of 'balanced' growth, i.e. all the molecular species of the cell are increasing at the same

rate and that there are no variations or changes in the cell population with growth rate (Bull, 1974).

Monods' recognition of the role of the limiting nutrient in the control of microbial growth was extended by Herbert, Elsworth, & Telling (1956) who perceived that control over the supply of the limiting-nutrient could be achieved through the system of cultivation. Novick and Szilard (1950; cited by Fiechter *et al.*, 1987) had already developed a way of establishing the continuous growth of a micro-organism as an equilibrium condition by ensuring that culture volume and cell density were kept constant by the continual addition of medium and removal of an equal volume of culture. Within the 'chemostat', the specific growth rate (μ) of the cells is fixed by the concentration of the limiting nutrient whose level is determined by the rate of addition (F) of the medium as a ratio of the culture volume (V) (Herbert, 1958). This ratio is termed the 'dilution rate', D, where $D = F/V$. For any given value of D, the system will self-regulate as a result of the nutrient-limitation until all variables remain constant despite the flux of materials and μ becomes equal to D (Herbert, 1964), i.e. a 'steady-state' is reached. This steady-state relates the conditions of culture directly to the growth rate of the organism and hence to the cell itself, a relationship absent during batch growth (Tempest, 1970). Hence, the systematic evaluation (Herbert *et al.*, 1956) of the effect of changes in growth rate on cell morphology, physiology and metabolism is possible.

Quantitative relationships, based on gross cellular properties such as mass, size, and age, for various organisms and limiting substrates were found to hold to the Monod model (Herbert, 1958). However, deviations from the original mathematical model were observed whereby qualitative changes occurring between specific growth rates under carbon-limitation (Herbert, 1961; Herbert *et al.*, 1956) were different to changes occurring under nitrogen-limitation (Tempest and Herbert, 1965; Herbert, 1961). These and other emerging differences, indicated that growth was not uniform over a range of growth rates, and were ascribed by Malek (1958; cited by Dawson, 1985) to the neglected importance of the 'physiological characteristics' of a culture. The idea of the

'physiological state' was introduced to encompass the non-quantitative, underlying metabolic and physiological aspects of growth (Malek, 1958; cited by Dawson, 1985) although the concept remains nebulous and poorly understood.

Bull (1974) reviews the potential reasons for non-ideal behaviour whilst Harrison and Topiwala (1974) observe that experimental data are rarely in accord with simple unstructured models of microbial behaviour and that the deviations observed "indicate complex regulatory mechanisms at several metabolic levels".

The dynamic behaviour of continuous cultures was reviewed by Harrison and Topiwala (1974) who propose the study of transient responses to perturbations of the steady-state as a way to examine the control mechanisms of a cell's metabolism that are not usually evident during steady-state measurements. Hence, the elucidation of unidentified components and biochemical pathways is possible by observing the transient fluxes of cell constituents as a response to the nutrient pulses (Bull, 1974). For example, the transition of *S. carlsbergensis* from aerobiosis to anaerobiosis (Pye, 1969; cited by Harrison & Topiwala, 1974) revealed a recovery of the system from the perturbation within minutes rather than hours that indicated an allosteric response rather than an induction or repression mechanism.

Steady-state responses to increases in the concentration of the carbon source were used to develop a technique for media optimization (Mateles and Battat, 1974). Other applications of the chemostat include studies of the importance of substrate concentration on enzyme systems (Fencl and Pazlarova, 1982; Matin, 1981; Bull and Brown, 1979; Melling, 1977; Sikyta and Fencl, 1976; Dean, 1972; cited by Dawson, 1985); regulatory phenomena (Dawes, 1981; Tempest and Neijssel, 1976; Tempest, Meers, & Brown, 1973; cited by Dawson, 1985); toxicity (Melling, 1977; cited by Dawson, 1985); nutrient uptake pathways, e.g. glutamate synthesis (Bull and Brown, 1979; Tempest *et al.*, 1973; cited by Dawson, 1985); and genetic aspects (Dykhuizen and Hartl, 1983; Calcott, 1981; cited by Dawson, 1985).

The function and occurrence of secondary metabolism have been investigated using the chemostat to explore the relationship between the limiting substrate and

growth rate of *Gibberella fujikuroi* where it was found that three different types of secondary carbon metabolites, normally suppressed during non-limited growth, were formed at differing degrees of growth limitation (Bu'Lock, Detroy, Hostlaek, Munim-al-Shakarchi, 1974; cited by Dawson, 1985). It was suggested (Bu'Lock, 1973; cited by Dawson, 1985) that the relevant genes are differentially sensitive to the levels of nutrient limitation that is itself an effector or index of a common mechanism of 'growth suppression' that acts as an overall regulating device to harmonize the activities of secondary biosynthesis with replicatory growth (Bu'Lock, 1975; cited by Dawson, 1985). The pattern of secondary metabolism is determined not only by the presence of a limit on replicatory growth but also by the intensity and nature of the limitation.

3.2.2. Chemostat Dilution Rates

3.2.2.1. Introduction

The establishment of a chemostat using magnesium as the limiting nutrient was seen to be a way of studying the yeast cell's morphology, physiology and metabolism at different levels of growth under steady-state conditions. According to the literature reviewed in section 3.2.1, this technique should circumvent the problems of reproducibility, reliability and interpretability associated with non-steady-state batch cultures (Meyer *et al.*, 1985). The assessment of the relationship between the physiological state of the cell population and the limiting nutrient included the monitoring of gross population properties such as biomass, cell numbers, cell size, and yield co-efficients as indicators of phenotypic activities; stoichiometric parameters as a way of determining the fate of the magnesium; and metabolic variables such as the rate of oxygen uptake, glucose consumption, and ethanol synthesis as indicators of respiratory and fermentative behaviour.

The objective of the experiments detailed below was the characterization of a population of magnesium-limited cells for a range of balanced growth rates. The expectation was that because growth was controlled by magnesium then the role of

magnesium within the cell would be reflected by the measurable characteristics of the cell population.

The results from the batch cultures (section 3.1.1) were used to estimate the concentration of magnesium within the inflowing medium necessary to limit growth of the cells within the chemostat. From these batch results, the linear decline in growth rate occurs below 250μM and therefore a magnesium concentration of 90μM within the chemostat medium was estimated to be sufficiently limiting. From the theory reviewed in section 3.2.1 it would be expected that the actual concentration of magnesium used to limit the growth of the population would be irrelevant provided the concentration is limiting, however, a lower concentration of limiting-magnesium (50μM) had to be used as 90μM was found to be insufficiently limiting.

3.2.2.2. Methods

The chemostat set-up has been detailed in section 2.2.1; the medium preparation in section 2.2.2; the inoculum preparation in section 2.2.3; and the sampling regime in 2.2.4. Analyses of samples have been covered in section 2.3.

Continuous culture was initiated as described in section 2.2.3 whereupon the chemostat was sampled at the lowest dilution rate of $0.05hrs^{-1}$ and then allowed to recover from the effect of sampling over a period of four chemostat volumes. A second sample was then taken and again a re-equilibration permitted before a third sample was removed from the chemostat whereupon the dilution rate was increased to $0.10hrs^{-1}$ and the system allowed to establish a new steady state. The sampling procedure was repeated at this new dilution rate and at the dilution rates of 0.15, 0.20, 0.25, and $0.30hrs^{-1}$.

This procedure was carried out using an exogenous magnesium concentration of 90μM within the medium reservoir and then repeated using a concentration of 60μM followed by a third trial at 50μM.

3.2.2.3. Results and Discussion

Magnesium Limitation at 50μM

The results detail several directly measured fermentative and respirative aspects of the continuous, magnesium-limited growth of a cell population of *S. cerevisiae* within a chemostat supplied with the minimal medium used during the batch studies (section 3.1.1). Also presented are several derived parameters calculated from these results. The results for the magnesium limitation at 60μM are not presented here as the trends are identical to those appearing at 50μM limitation.

Changes in the measured parameters (cell concentration, medium absorbance, cell dry weight, mean cell volume, medium ethanol, glucose, and magnesium, cellular magnesium levels, oxygen uptake rate, medium pH and oxygen levels) as a function of the magnesium-controlled growth rate are shown in Figures 3.30, 3.31, 3.32, 3.33, 3.34, 3.35, 3.36, 3.37, 3.38, 3.39, 3.40, 3.41, 3.42, and 3.43 respectively. The effect of growth rate on the derived variables of biomass and ethanol yields (for glucose and magnesium), ethanol production and glucose uptake are shown in Figures 3.44, 3.45, 3.46, 3.47, 3.48, and 3.49 respectively.

In the interpretation of chemostat data it is necessary to utilise the principle derivations from the batch studies (Bull, 1974); namely the maximal growth rate (μ_{max}), the substrate saturation constant (K_S), and the growth yield (Y). Of these, μ_{max} provides a reference point from which to interpret the continuous culture results whereby at dilution rates greater than μ_{max} ($0.200 hrs^{-1}$) washout is expected and at dilution rates less than μ_{max} deviations from the theoretical curves are more easily identifiable.

The primary observation is that the cell population is magnesium-limited as indicated by the low residual exogenous magnesium within the chemostat (Figure 3.36) relative to the magnesium concentration within the reservoir magnesium (Figure 3.37). To support this conclusion, Figure 3.35 indicates that the culture is not glucose-limited

at any dilution rate tested nor is oxygen a limiting factor (Figure 3.43) although the medium is strongly depleted of oxygen at the higher growth rates.

From the premise that the cells are in a state of magnesium-limited growth, the second observation is that the curves for cell concentration (Figure 3.30), optical density (Figure 3.31) and cell dry weight (Figure 3.32) do not follow the theoretical patterns expected (Herbert, 1958) but do bear a close resemblance to documented divergences.

Up to μ_{max}, the trend of these graphs indicates that the biomass yield (magnesium) is not constant but actually increases as the growth-rate decreases. This is as expected from the yield data obtained during the batch studies (Figure 3.38) where the yield exponentially increased as the initial magnesium concentration was lowered. This increase was paralleled by a concomitant decrease in the growth rate. The trend is more easily identified in Figure 3.46 where it can be seen that the biomass yield co-efficient for magnesium is high at the lower dilution rates but becomes constant as the growth rate increases; again reflecting batch data and in keeping with the observations of other authors (Vrana, 1983). The ethanol yield co-efficient for magnesium (Figure 3.47) repeats this pattern, again in keeping with the batch results, but the curve is more exaggerated. The decrease between the ethanol yield at low growth rates and at high growth rates is a factor of three whereas the decrease for the biomass yield is only a factor of two.

This behaviour was first seen in *E. coli* growing under ammonium-limitation (Holme, 1957) and was subsequently observed for *Torula utilis* under ammonium-limitation (Herbert, 1958). Herbert's interpretation of this type of divergence was that the yield (weight of cells formed per weight of substrate used) is not constant but increases as the growth rate decreases and it is now believed that the utilization of the substrate in the production of intracellular reserves such as glycogen, polyhydroxybutyrate and volutin gives rise to this effect, especially when growth is other than carbon-limited (Bull, 1974).

The low residual glucose levels (Figure 3.35) within the chemostat at low dilution rates strengthens this argument based on the premise that the cells are converting the extra glucose removed from the medium at the lower dilution rates into reserve materials. This argument is fortified by Figure 3.44, where at the lower dilution rates under discussion, the biomass yield co-efficient for glucose is not only relatively low but also remains constant. Further, the glucose consumption (Figure 3.49) is high at the low dilution rates possibly indicating that the Pasteur effect is operational within the cells.

The high ethanol levels within the chemostat at the low dilution rates (Figure 3.34) indicate the activity of fermentative metabolism as do the high residual oxygen levels (Figure 3.43) and the low oxygen uptake rate on a 'per cell' basis (Figure 3.40) and as a ratio of dry weight (Figure 3.41). However, as the growth rate increases, the cells appear to increase their level of respiratory activity as indicated by the increase in the oxygen uptake rate, the parallel decrease in residual oxygen within the chemostat, and the reduced ethanol production levels (Figure 3.48).

The trend appears to be that the cells are primarily fermenting at low growth rates under magnesium-limitation but introduce a greater degree of respiratory activity as the growth rate increases towards μ_{max}. The results from the batch studies (section 3.1.1) are in agreement whereby the cells incapable of growth at low levels of magnesium (15μM) were still producing ethanol.

Figures 3.44 and 3.45 show that at the higher dilution rates, the actual conversion of glucose to biomass and ethanol both become more efficient but it is of note that the biomass yield increases by a factor of 4.75 whereas the ethanol yield only increases by a factor of 2.5, again indicating an increase in respiratory activity. It is of significance that both yield co-efficients for glucose only start to increase when the magnesium yield co-efficients (Figures 3.46 and 3.47) begin to level out; the interpretation being that at the lower growth rates the utilization of glucose has been 'damped' by the shortage of magnesium but the yield co-efficients for magnesium are high at these lower growth

rates as an efficiency of utilization is necessary. However, at the higher growth rates, as the cells are washed out and the magnesium is no longer limiting there is a more efficient utilization of the glucose coupled with a less efficient utilization of magnesium. It has been shown that increases in the concentration of *Bacillus subtilis* grown in magnesium-limited chemostats results in an enhanced assimilation of magnesium ions (Bull, 1974) possibly due to the secretion of an uptake-promoting substance and it is possible that such an event is taking place here.

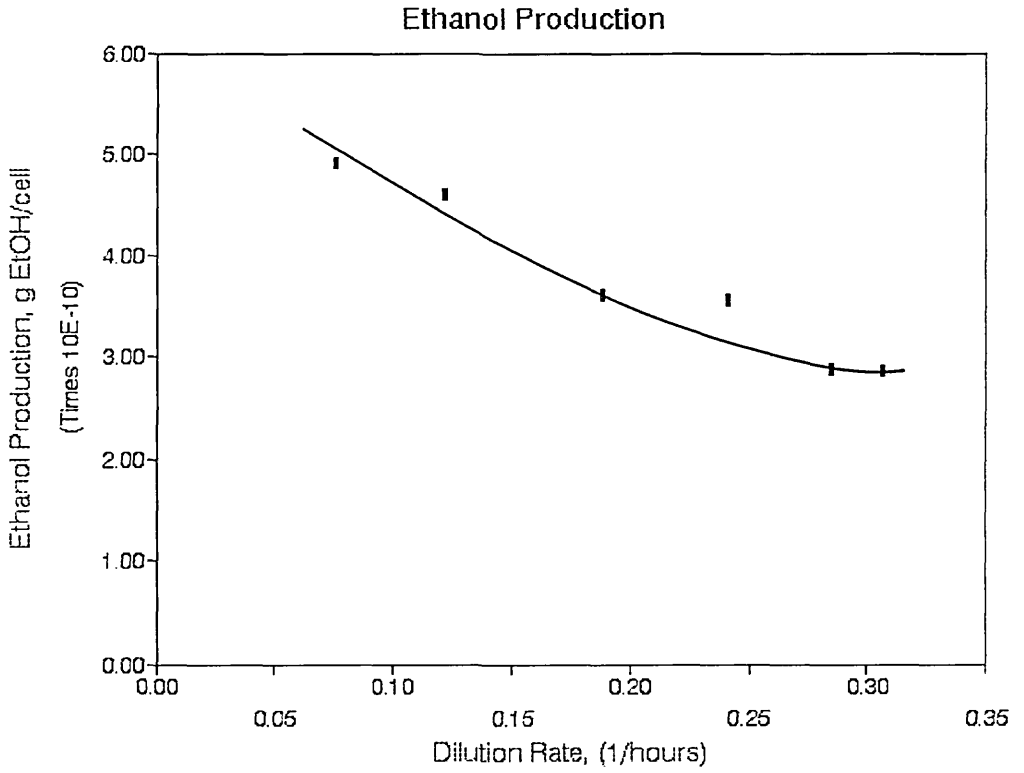
James (1961) recommends that data obtained from continuous culture studies be expressed 'per average cell', a view that is reinforced by Dawson (1985) who states that because cell populations alter their numbers with growth rate, the nutrient ratio per cell will change and that metabolic assessments made on the basis of mass differ to calculations based on the cell, indicating that Monod's assumption of the uniform development of the cell is incorrect. Further, cell concentration reveals numerical changes indicative of cell division that is tied to population cell growth whereas optical density indicates biomass changes that are tied to individual cell growth and that whilst quantitative assessments may be made using mass calculations they tend to obscure the qualitative changes occurring within the cell (Dawson, 1985).

The data for cellular magnesium and oxygen uptake rates are presented as a function of cell dry weight and per unit cell (Figures 3.38 and 3.39; 3.40 and 3.41) but no significant difference between the two modes of expression is seen whilst biomass yield, by definition, is expressed as cell dry weight.

The ethanol production values based on cell dry weight (Figure 3.48), as mentioned, indicate that as the dilution rate increases the predominance of fermentation decreases. To confirm this, ethanol production values based on cell numbers were calculated and are presented in Figure 3.50 below. Again, the results show that there is no significant difference between the two modes of expression as well as confirming

that the cells favour fermentation at low growth rates but reduce this activity as maximal growth is approached.

Figure 3.50: The influence of increasing growth rate on the ethanol production per cell within a magnesium-limited (50μM) chemostat.



Obviously, of prime importance throughout all of these observations are the trends in cellular magnesium patterns (Figures 3.38 and 3.39). Evidently, at the low growth rates where fermentation predominates, the cellular concentration of magnesium is lowest but as the growth rate increases then the cellular magnesium concentration also increases. This trend is observed for cellular magnesium expressed either on a per-cell basis or on a gram of dry weight basis thus lending support to the idea expressed above, namely that the increased availability of magnesium permits the general utilization of glucose to become more efficient but with greater emphasis on the respiratory pathway. This increase in the cellular content of magnesium with increasing

growth rate parallels the situation in bacteria (Dawson, 1985) whilst Dedyukhina, Chistyakova, Eroshin, & Kashparova, (1989) observed that a magnesium-limited chemostat of *C. valida* gave a linear rise in the cellular content of P, K, Mg, N, Fe, and Zn with increasing growth rates.

All the figures refered to have shown data for growth greater than the maximal growth rate derived from the batch studies for magnesium-limitation. In fact, it is at μ_{max} that the changes become the most exaggerated; with the cell concentration dropping more rapidly; the decrease in dry weight ceasing; the mean cell volumes beginning to decline; the biomass and ethanol yield coefficients for glucose increasing sharply and the yields for glucose becoming constant as do the cellular magnesium levels.

This type of growth curve where the organism is growing faster in continuous culture than in batch culture is recorded in the literature as being due to imperfect mixing within the chemostat (Bull, 1974 and Herbert, 1958) principally observed for filamentous microbes which adhere to the fermenter walls and are not properly washed out.

During the progression of the sequential increase in dilution rates the cells within the chemostat were observed to undergo a progressive change in morphology from the characteristic ovoid shape to a more extended form as μ_{max} was reached and passed. This extended form appeared to be more prevalent in the daughter cells giving the impression of pseudo-hyphal growth and it was also noted that the separation of daughter cells from the mother cells seemed to decrease giving rise to clumps of cells all apparently still connected. These clumps also increased in size and although attempts were made to measure the progressive increase in clump size using the coulter counter these proved unsuccessful due to the tendency of the clumps to float. Sedimentation tests were carried out on the clumps whereby 10mls samples were centrifuged, rinsed, recentrifuged and the cell pellets resuspended in 10mls acetate buffer at pH 4.6 which was then decanted into graduated test-tubes. The degree of

sedimentation after 10mins was found to be considerably less than for a suspension of cells taken from the inoculating medium with many of the cells remaining in a white floc on the surface. Overnight incubation in the presence of excess magnesium did not cause a reversion of the clumps nor was the sedimentation rate affected.

Figure 3.51 shows photographs of the progression of change occurring to the cells at a dilution rate of $0.25hrs^{-1}$. Photograph a) shows individual cells beginning to elongate; photograph b) shows the lack of separation taking place; photograph c) shows clump formation beginning to occur; and photograph d) shows clump of cells, the like-of-which came to predominate within the chemostat at the highest growth rates; i.e. after long periods of culture within the chemostat. Changes in the morphology of *S. cerevisiae* with dilution rate have been observed previously (Fiechter, 1967; cited by Fiechter *et al.*, 1987) whilst Botstein and Fink (1988) propose that *S. cerevisiae* is a truly dimorphic and/or polymorphic yeast.

Figure 3.30: The influence of dilution rate on the cell concentration within a chemostat operated under conditions of magnesium-limitation (50uM).

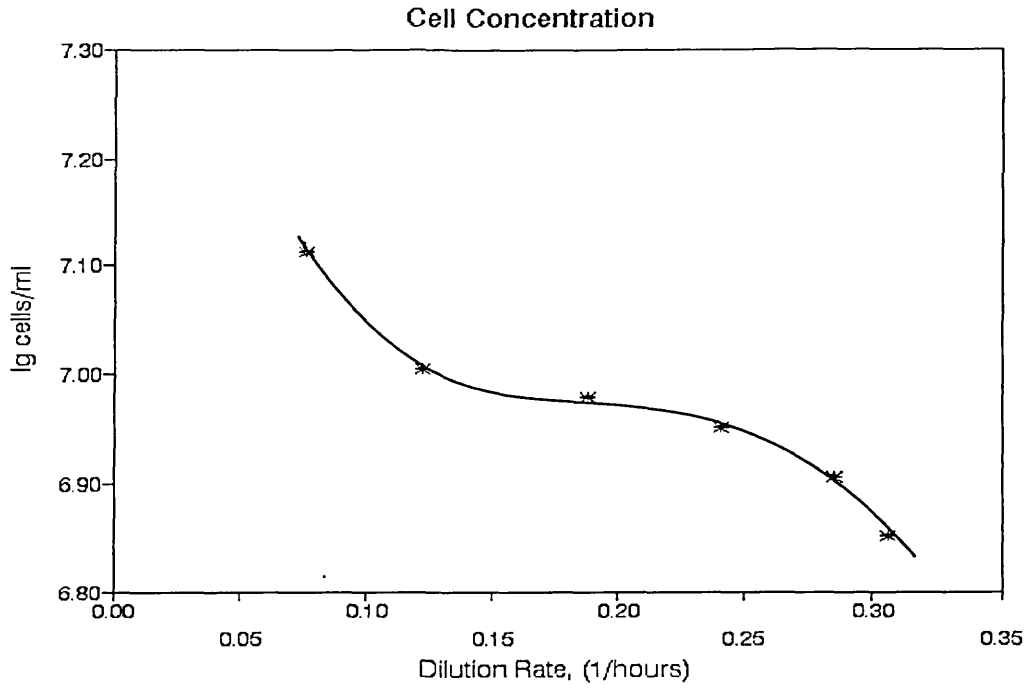


Figure 3.31: The influence of dilution rate on the optical density within a chemostat operated under conditions of magnesium-limitation (50uM).

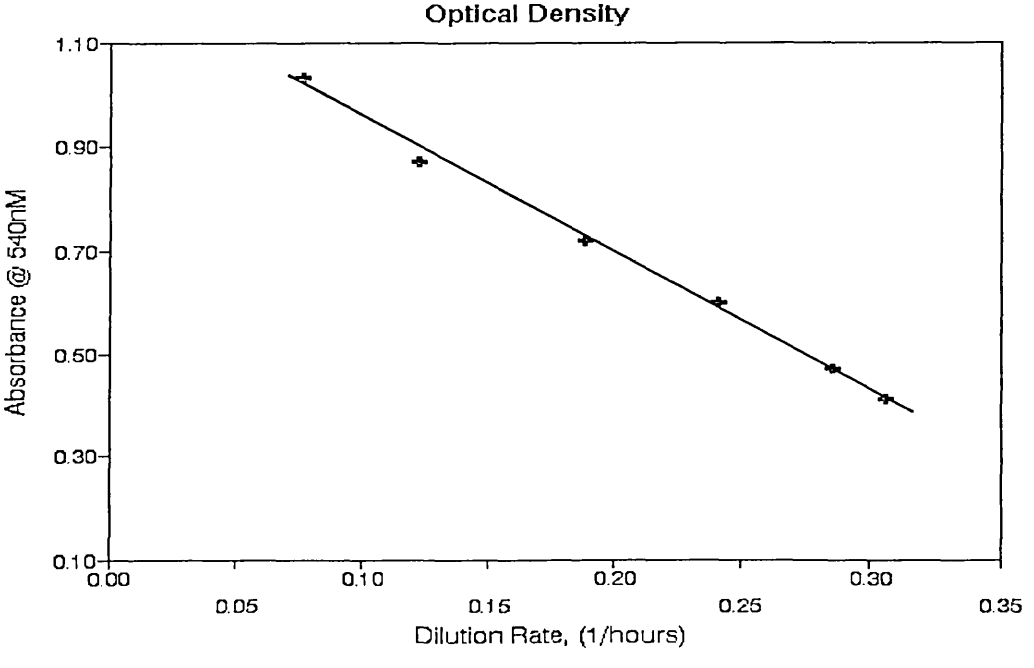


Figure 3.32: The influence of dilution rate on the dry cell weight within a chemostat operated under conditions of magnesium-limitation (50uM).

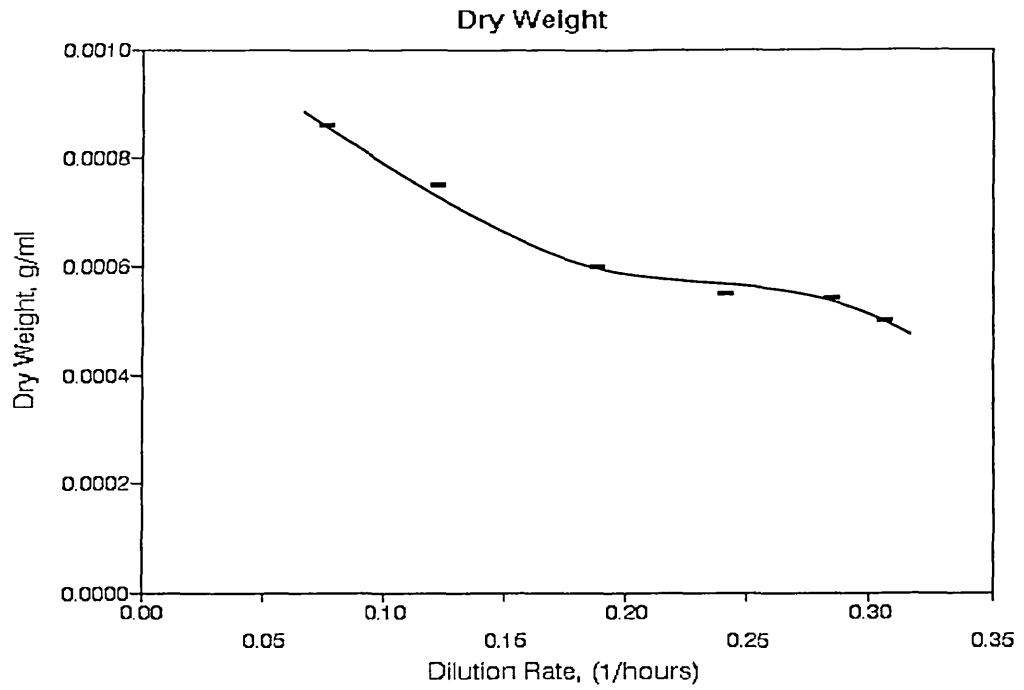


Figure 3.33. The influence of dilution rate on the mean cell volume within a chemostat operated under conditions of magnesium-limitation (50uM).

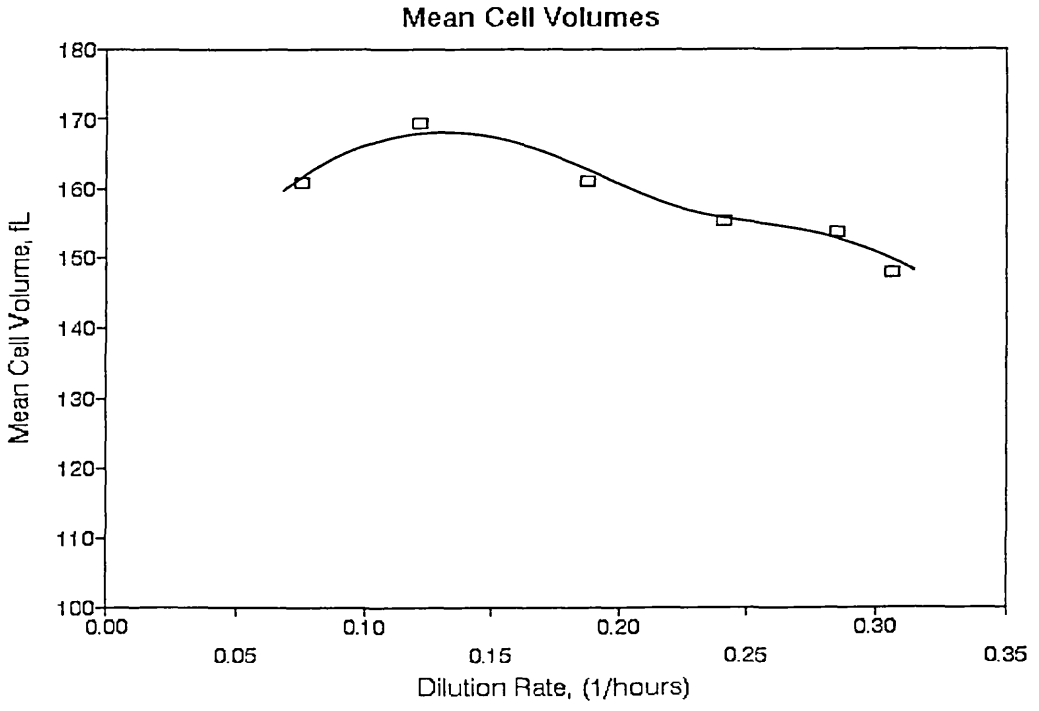


Figure 3.34: The influence of dilution rate on the ethanol concentration within a chemostat operated under conditions of magnesium-limitation (50uM).

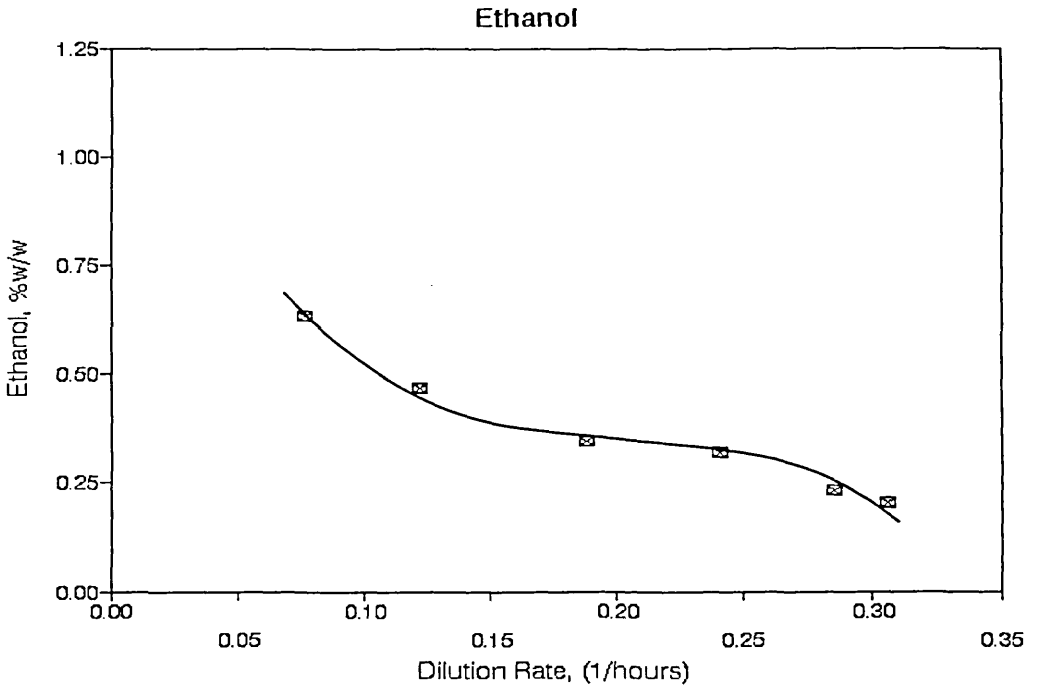


Figure 3.35: The influence of dilution rate on the glucose concentration within a chemostat operated under conditions of magnesium-limitation (50uM).

Figure 3.36: The influence of dilution rate on the magnesium concentration within a chemostat operated under conditions of magnesium-limitation (50uM).

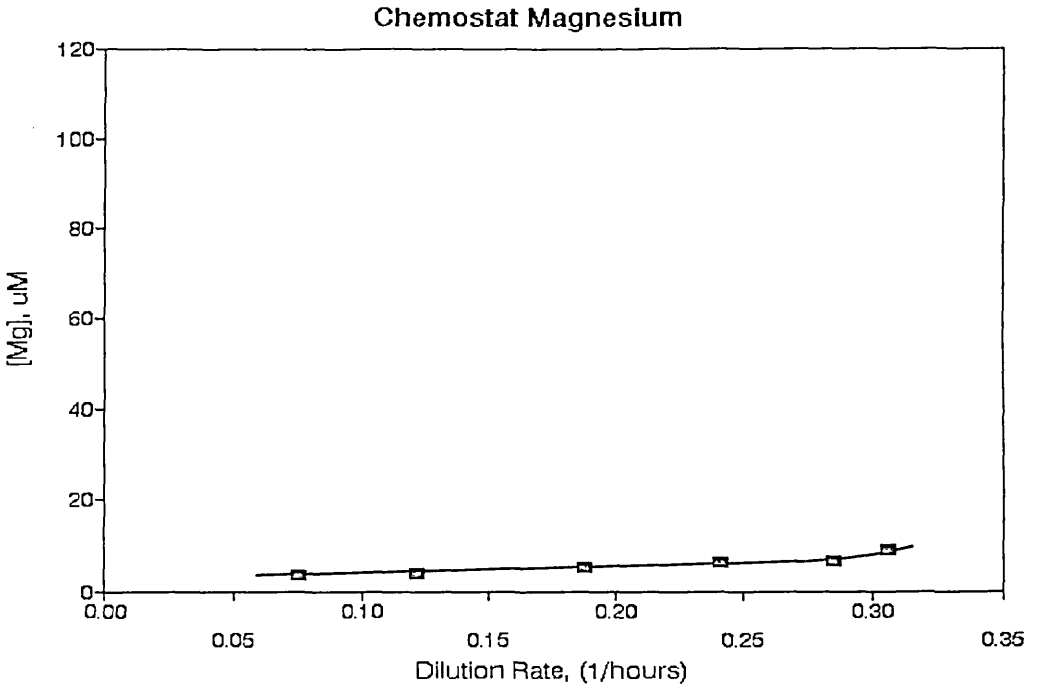


Figure 3.37: The magnesium concentration within the reservoir medium during the operation of a chemostat over a range of dilution rates.

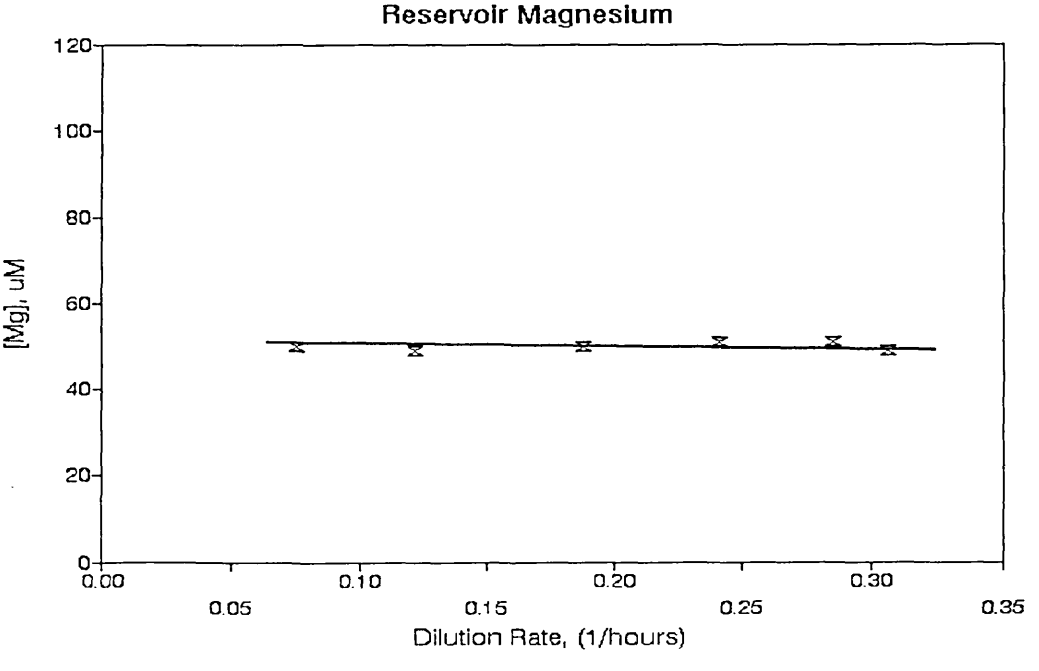


Figure 3.38: The influence of dilution rate on the cell magnesium concentration, expressed as a ratio of cell concentration, within a chemostat operated under conditions of magnesium-limitation (50uM).

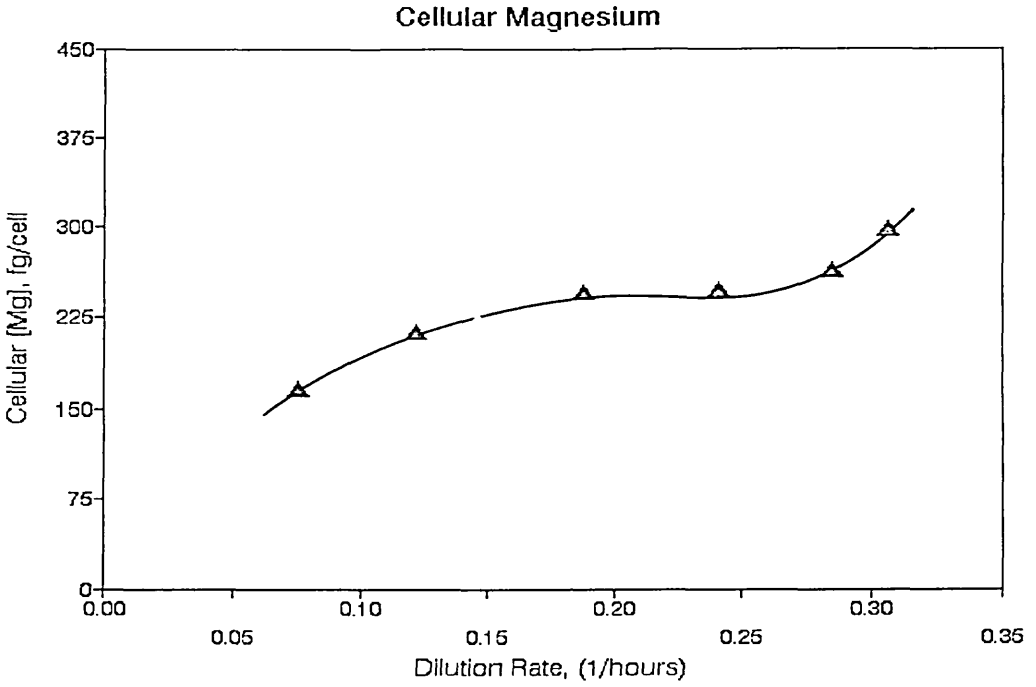


Figure 3.39: The influence of dilution rate on the cell magnesium concentration, expressed as a ratio of dry weight, within a chemostat operated under conditions of magnesium-limitation (50uM).

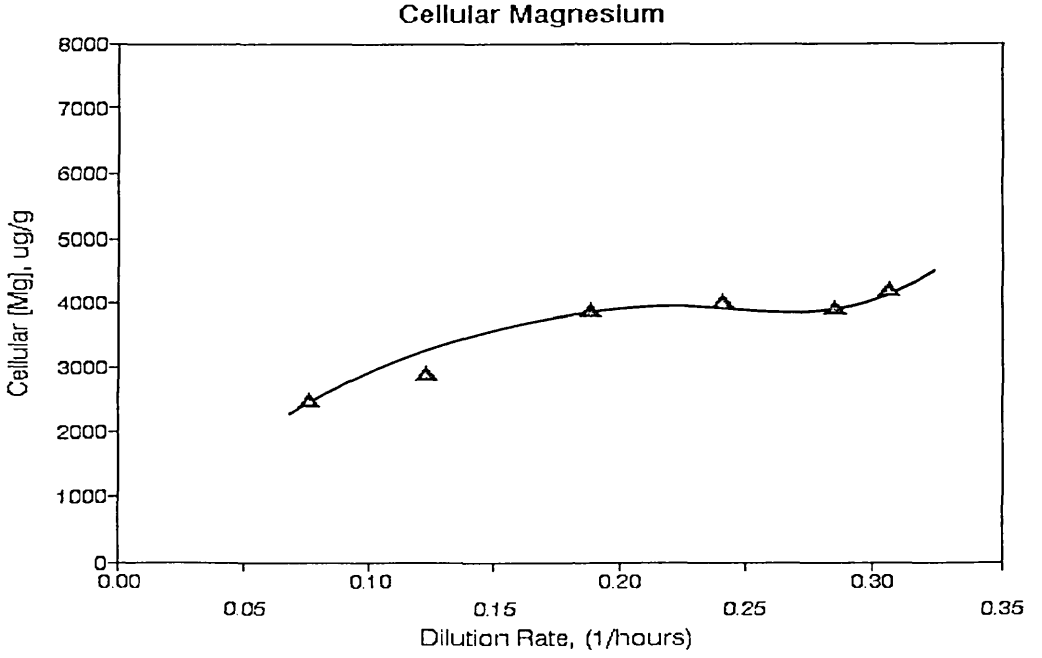


Figure 3.40: The influence of dilution rate on the oxygen uptake rate, expressed as a ratio of cell concentration, within a chemostat operated under conditions of magnesium-limitation (50uM).

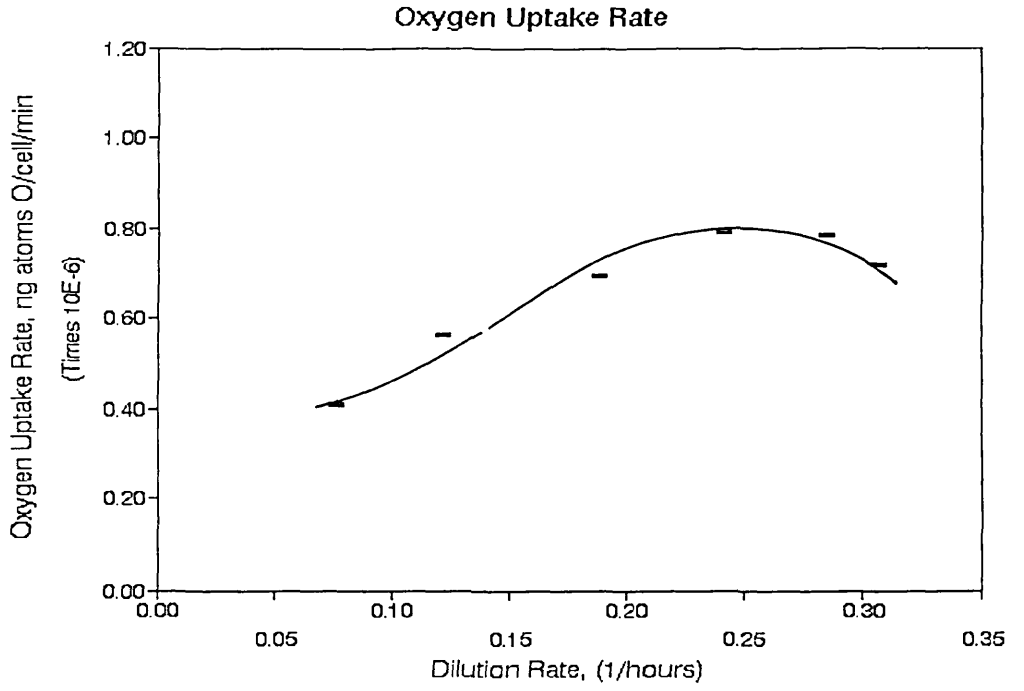


Figure 3.41: The influence of dilution rate on the oxygen uptake rate, expressed as a ratio of dry cell weight, within a chemostat operated under conditions of magnesium-limitation (50uM).

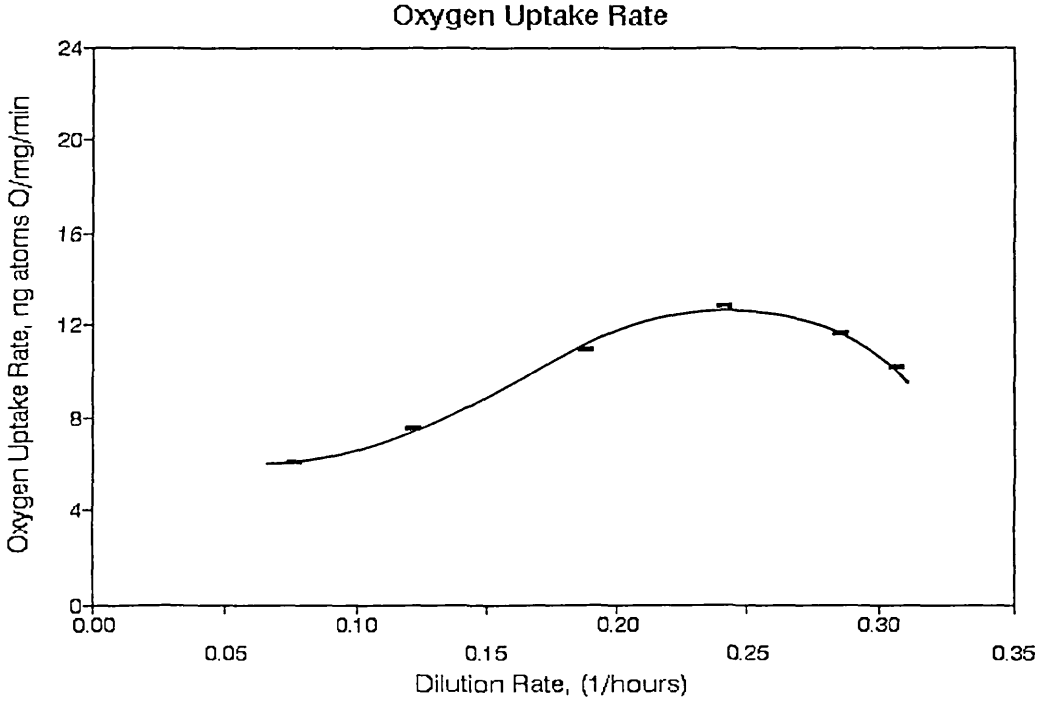


Figure 3.42: The influence of dilution rate on the pH within a chemostat operated under conditions of magnesium-limitation (50uM).

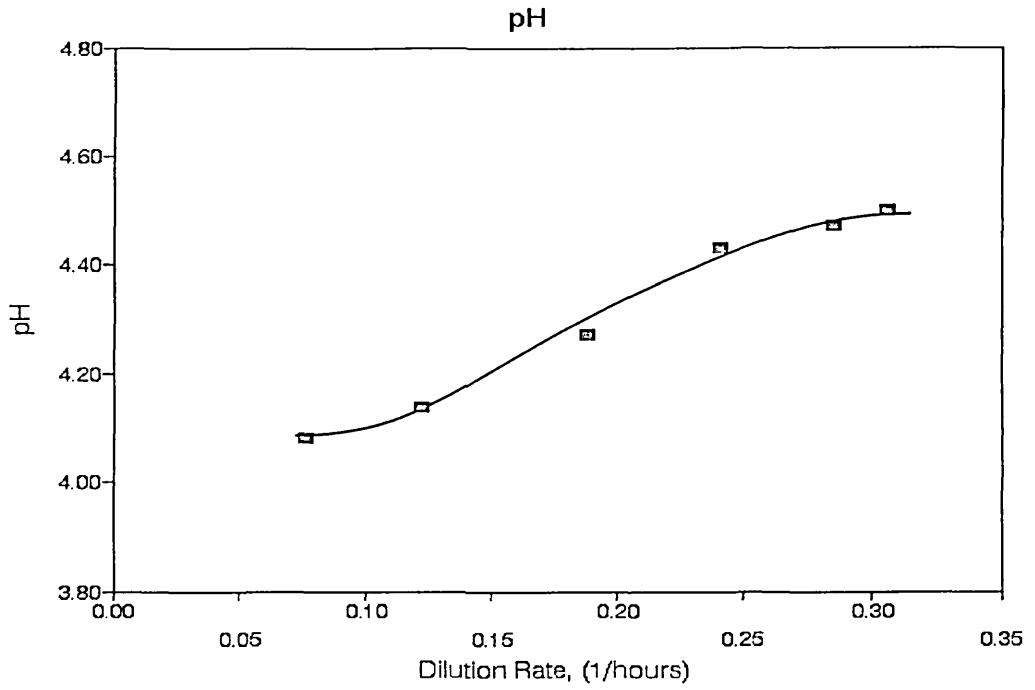


Figure 3.43: The influence of dilution rate on the oxygen levels within a chemostat operated under conditions of magnesium-limitation (50uM).

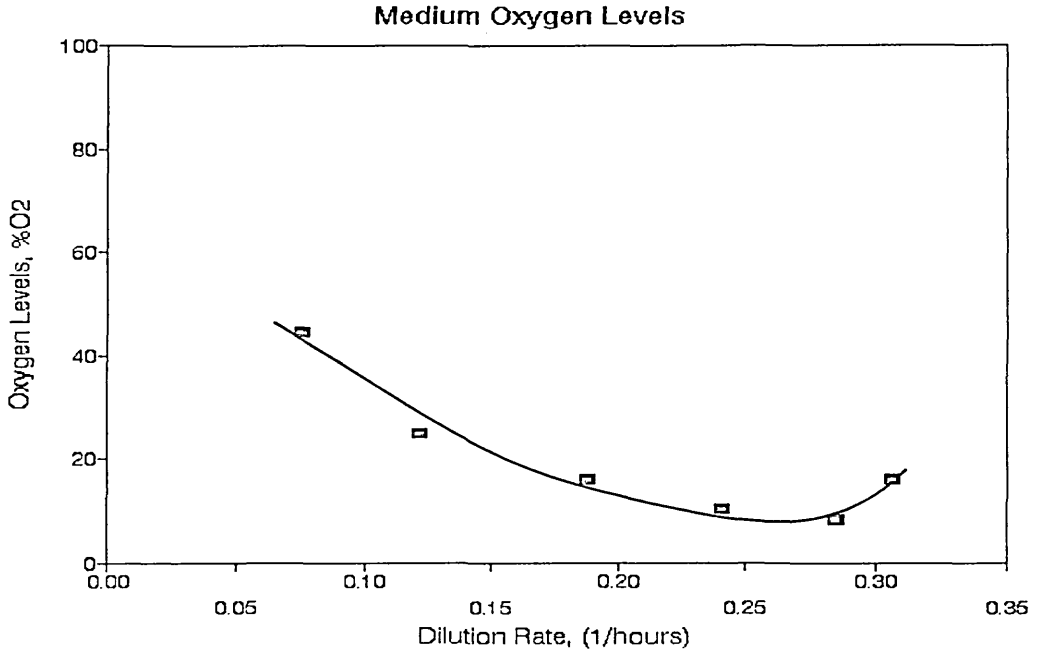


Figure 3.44: The influence of dilution rate on the biomass yield (glucose) within a chemostat operated under conditions of magnesium-limitation (50uM).

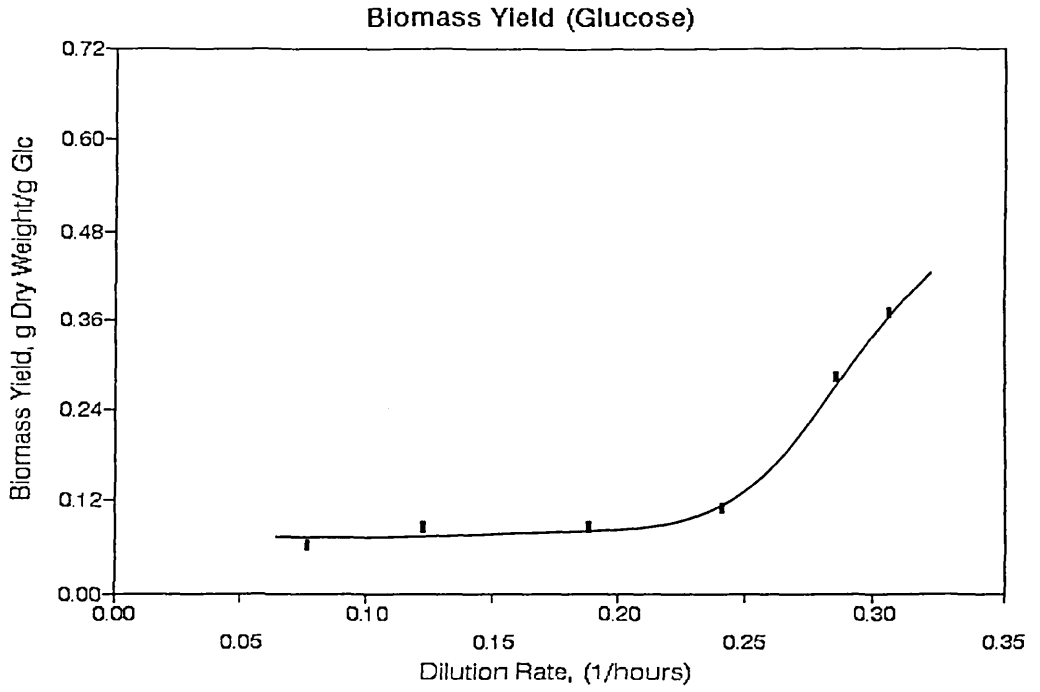


Figure 3.45: The influence of dilution rate on the ethanol yield (glucose) within a chemostat operated under conditions of magnesium-limitation (50uM).

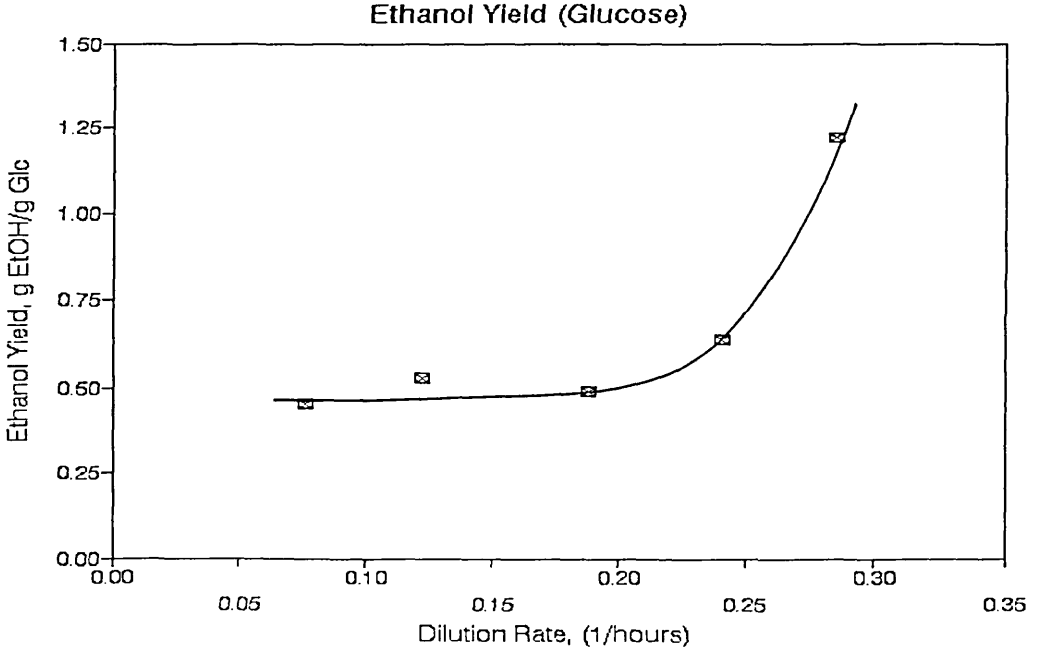


Figure 3.46: The influence of dilution rate on the biomass yield (magnesium) within a chemostat operated under conditions of magnesium-limitation (50uM).

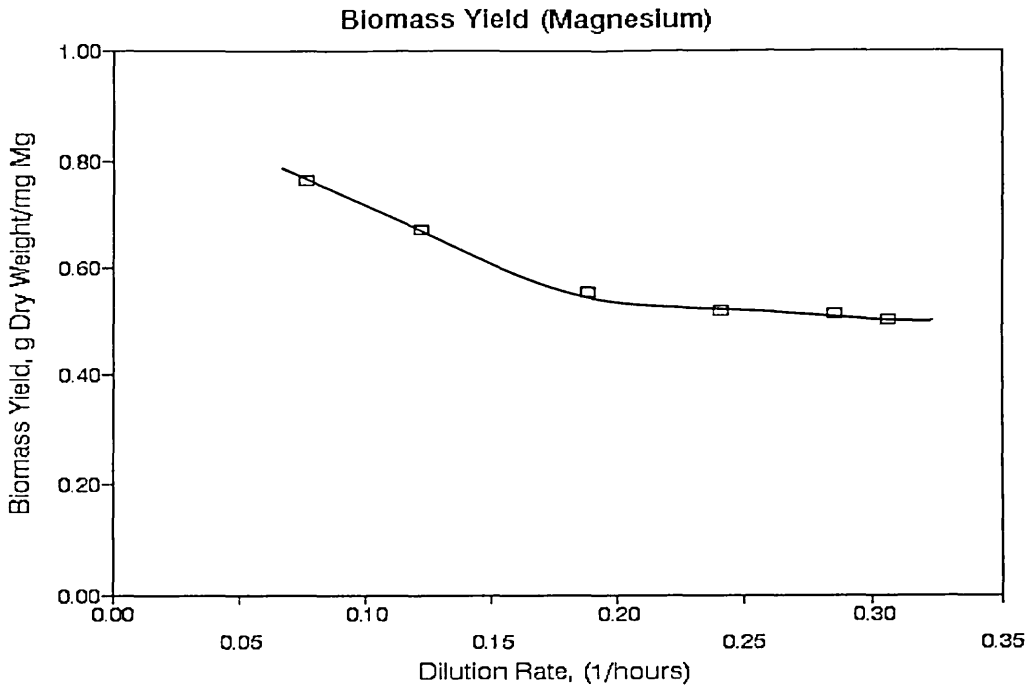


Figure 3.47: The influence of dilution rate on the ethanol yield (magnesium) within a chemostat operated under conditions of magnesium-limitation (50uM).

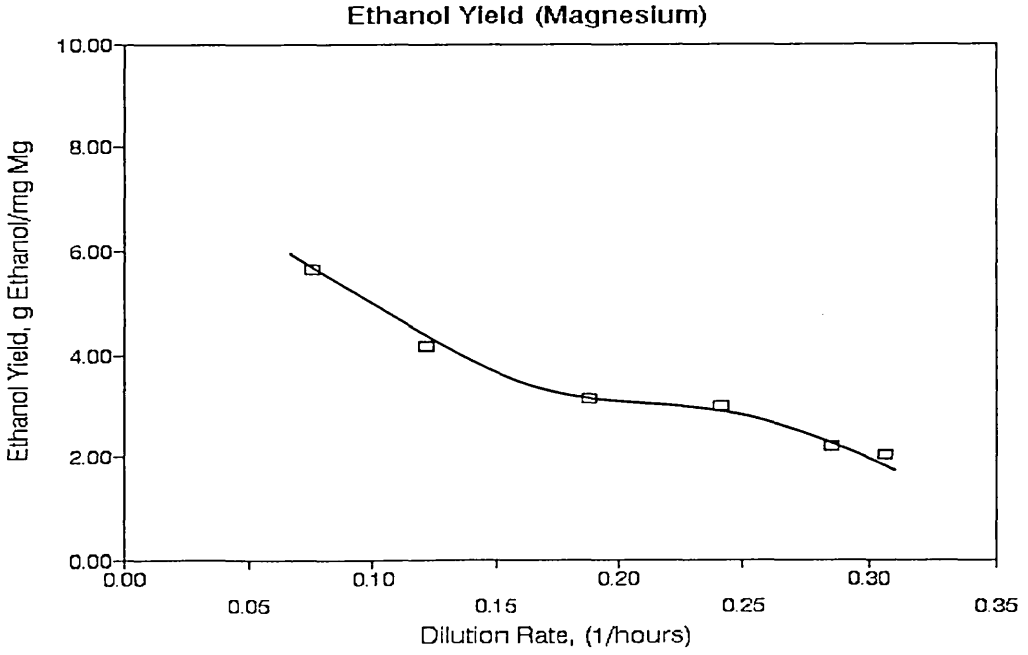


Figure 3.48: The influence of dilution rate on the ethanol production within a chemostat operated under conditions of magnesium-limitation (50uM).

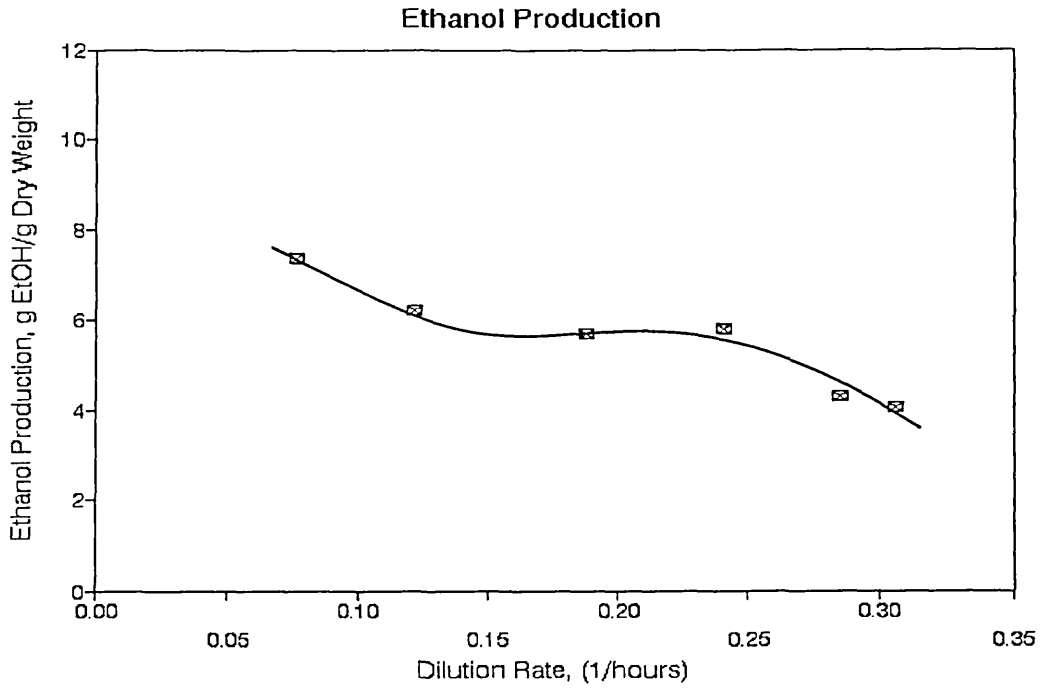


Figure 3.49: The influence of dilution rate on the glucose consumption within a chemostat operated under conditions of magnesium-limitation (50uM).

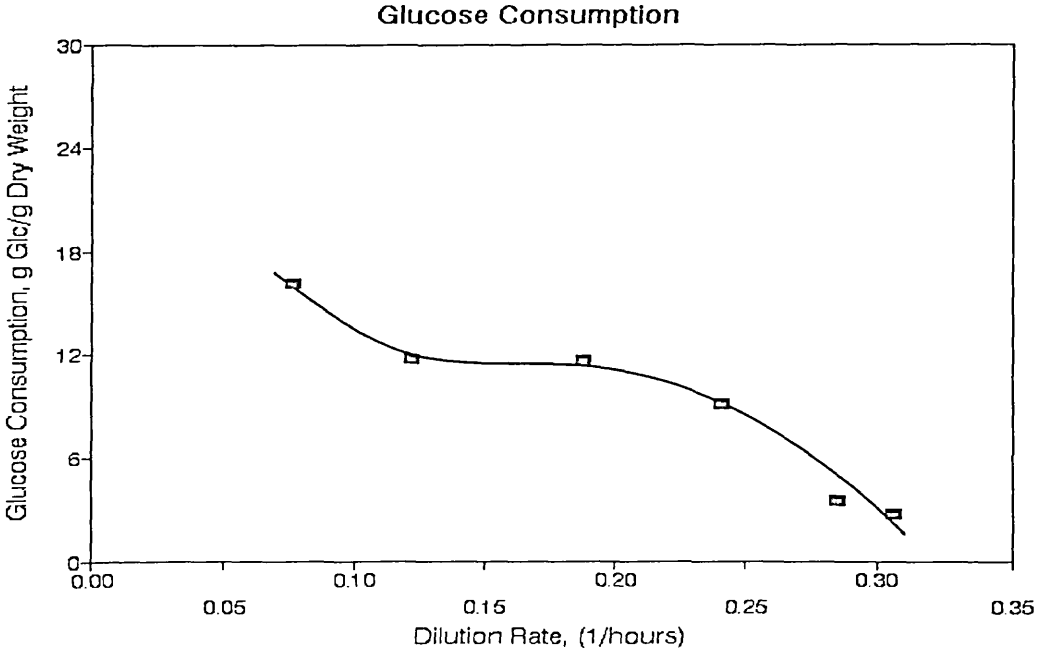
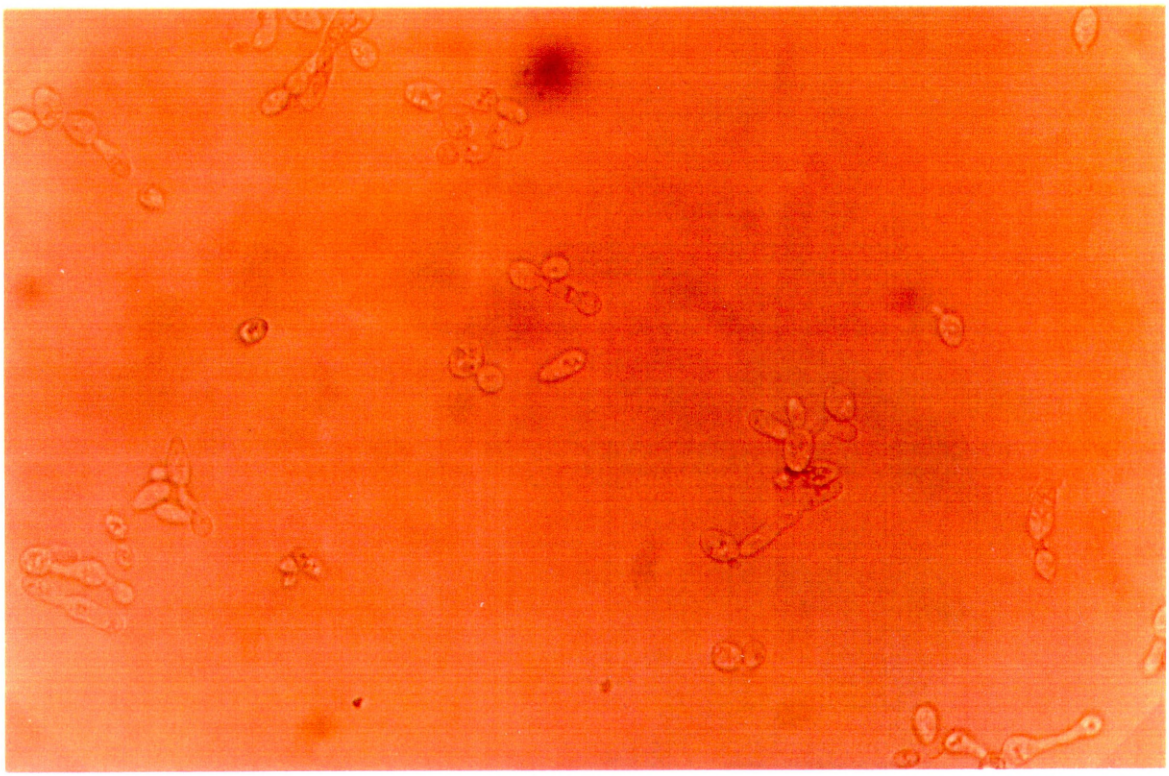


Figure 3.51: The progressive change in morphology of cells of *S.cerevisiae* cultured under magnesium-limitation at a growth rate of 0.25/hrs. Photographs a) and b). Magnification x400

Photograph a) Onset of tube-formation in individual cells



Photograph b) Lack of separation taking place between mother and daughter cells

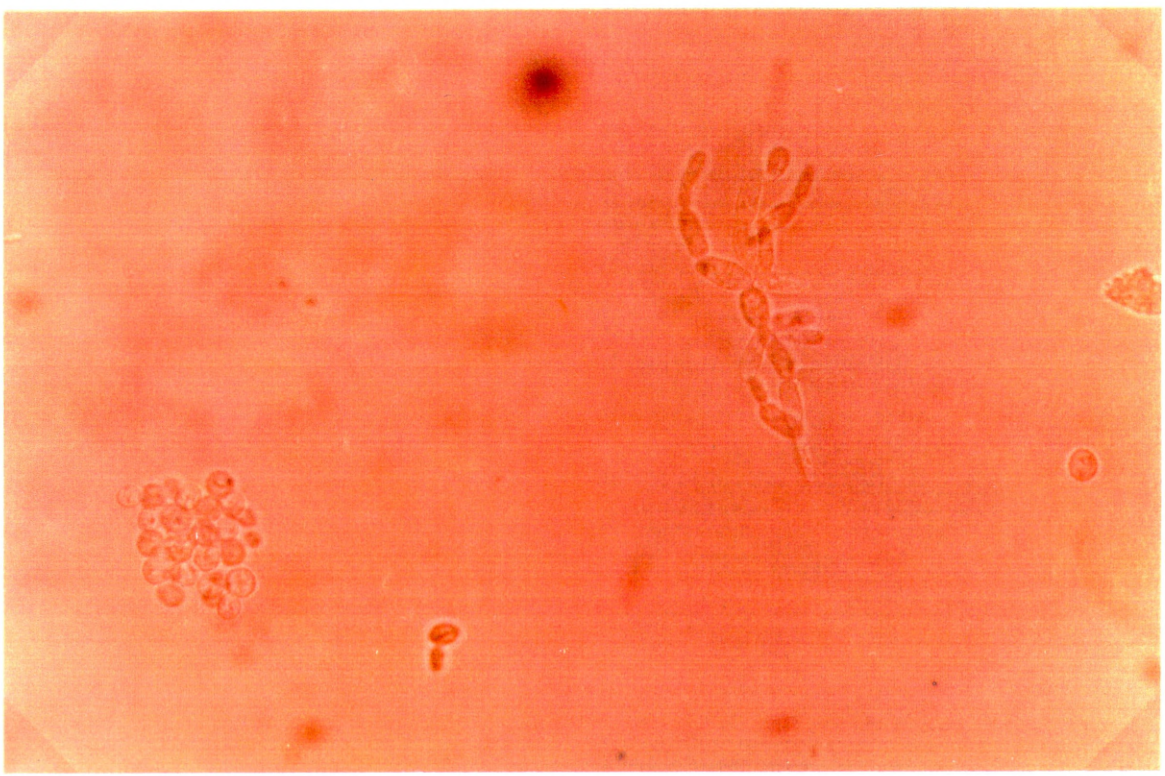
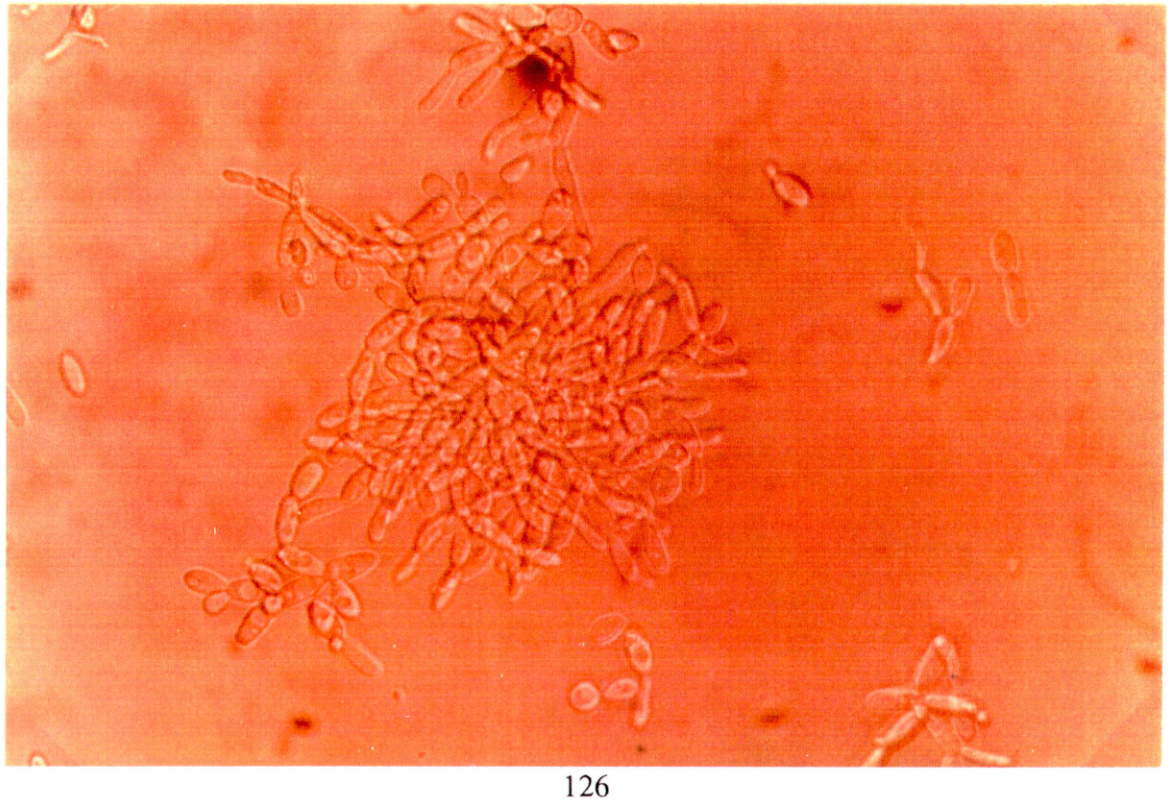


Figure 3.51 (continued): The progressive change in morphology of cells of *S.cerevisiae* cultured under magnesium-limitation at a growth rate of 0.25/hrs. Photographs c) and d). Magnification x400

Photograph c) Onset of clump-formation

Photograph d) Major clump formation showing pseudo-hyphal growth



Magnesium Limitation at 90μM

The results again cover the directly measured fermentative and respirative parameters of a continuous, magnesium-limited growth of a cell population of *S. cerevisiae* within a chemostat. Calculated parameters derived from the directly measured data are also detailed.

The measured parameters (cell concentration, medium absorbance, cell dry weight, mean cell volume, medium ethanol, glucose, magnesium levels, cellular magnesium levels, oxygen uptake rate, and pH), as a function of the magnesium-controlled growth rate, are shown in Figures 3.52, 3.53, 3.54, 3.55, 3.56, 3.57, 3.58, 3.59, 3.60, 3.61, 3.62, 3.63, and 3.64 respectively. No data for oxygen levels is available. The effect of growth rate on the derived variables of biomass and ethanol yields (glucose and magnesium), ethanol production (cell and dry weight) and glucose uptake (cell and dry weight) are shown in Figures 3.65, 3.66, 3.67, 3.68, 3.69, 3.70, 3.71, and 3.72 respectively.

Contrary to the expectations derived from batch results, the residual magnesium levels within the chemostat (Figure 3.58) appear to indicate that the cell population is not magnesium-limited when an exogenous magnesium concentration of 90μM is present within the inflowing medium (Figure 3.59). Mateles and Battat (1974) state that residues of the limiting substrate indicate some other factor has become limiting and the lack of residual glucose within the chemostat at the lower dilution rates (Figure 3.57) may indicate glucose-limitation.

From Figures 3.52 and 3.53, again a deviation from the expected theoretical curve (Herbert, 1958) for cell concentration and absorbance can be seen with significant changes in the trends appearing at $0.2hrs^{-1}$. However, unlike the behaviour observed for a magnesium-limitation of 50μM, Figure 3.54 shows that as the growth rate increases the cell dry weight also increases markedly. Hence, it would appear that as the dilution rate increases, the cells are not subjected to any apparent limitation and are

therefore growing individually, i.e. in terms of biomass, in an unrestricted manner with the population in terms of cell numbers being subject to the dynamics of the chemostat.

This interpretation is further indicated by the apparent differences that exist between the cellular magnesium levels when expressed on a per cell basis or when expressed as a function of cell dry weight (Figures 3.60 and 3.61). The same trend is observed for the oxygen uptake rate (Figures 3.62 and 3.63), ethanol production (Figures 3.69 and 3.70), and glucose uptake (Figure 3.71 and 3.72). The curves for the biomass and ethanol yield co-efficients for magnesium (Figures 3.67 and 3.68) confirm the trends indicated by the dry weight values and therefore further indicate an absence of magnesium-limitation.

As stated above, at growth rates less than $0.20hrs^{-1}$, the residual glucose levels within the chemostat indicate a glucose-limitation but despite the apparent lack of magnesium-limitation on growth, several trends emerge from these results.

On a per cell basis, at the lower dilution rates, the ethanol levels are uniformly high (Figure 3.56), the oxygen uptake rate is low as is the cellular magnesium content. From the results of the 50μM limitation (section 3.2.2.3: magnesium limitation at 50μM) this would infer that the individual cells within the population are fermenting. The low residual glucose levels (Figure 3.57), low ethanol yield from glucose (Figure 3.66), low biomass yield from glucose (Figure 3.65), and low cell dry weight (Figure 3.54) also indicate a relatively low conversion of glucose to biomass.

However, on a dry weight basis, at the low dilution rates, the population appears to be less fermentative as indicated by the high cellular magnesium content and the high oxygen uptake rate. These contradictory results become more confusing when it is appreciated that the ethanol production and glucose consumption (Figures 3.70 and 3.72 respectively) are relatively high at the lower dilution rates irrespective of the calculation method. This would infer that the cell population is indeed showing a predominance of fermentation at the lower dilution rates with a tendency towards an increased level of respiratory behaviour as the dilution rate exceeds μ_{max} and pseudo-hyphal growth

predominates. Also, the 10-fold increase in the biomass yield from glucose (as opposed to only a 2-fold increase in the ethanol yield), would indicate that the population is becoming more respirative and/or less fermentative.

One possible deduction to account for the differences between the oxygen uptake rate on a dry weight basis and the rate calculated per cell is that the cells are becoming more respirative (or less fermentative) as the growth rate is increased but the increase in cell bulk means that the relative proportion of respiratory machinery is decreasing; the inference being that the actual respiratory matter must remain constant within the cell irrespective of the cell's bulk. Hence, the activities of the respiratory matter within the cell can increase but not the actual quantities.

However, there is a degree of ambiguity over any conclusions drawn from this set of data as the cells within the chemostat were not thoroughly magnesium-limited but were rather glucose-limited with possibly a degree of magnesium-limitation at the lower growth rates occurring as well.

Figure 3.52: The influence of dilution rate on the cell concentration within a chemostat operated under conditions of magnesium-limitation (90uM).

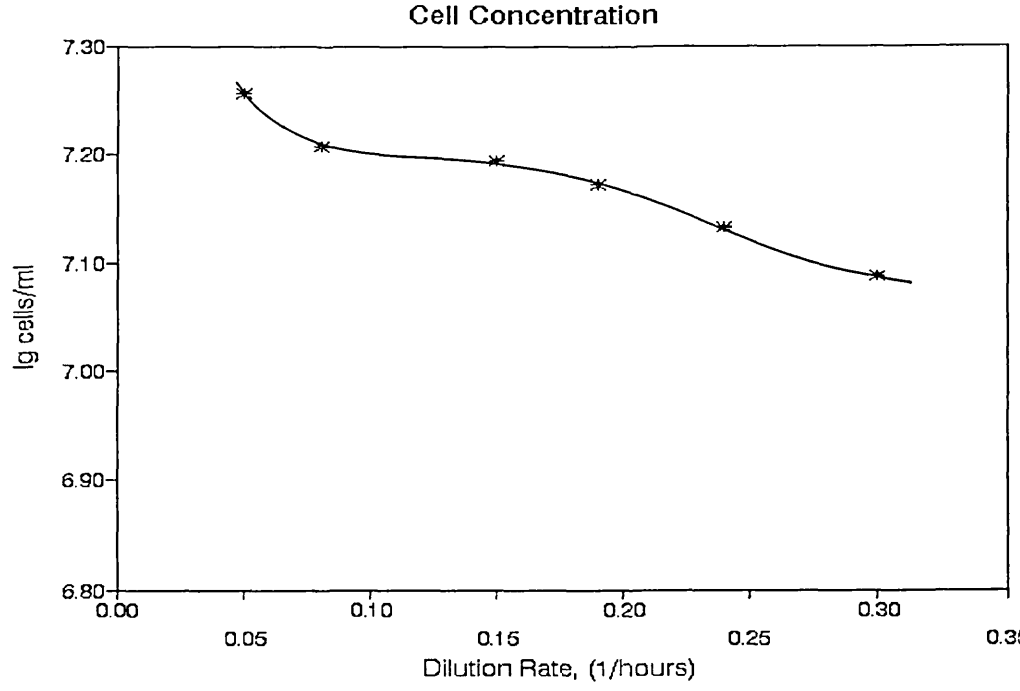


Figure 3.53: The influence of dilution rate on the optical density within a chemostat operated under conditions of magnesium-limitation (90uM).

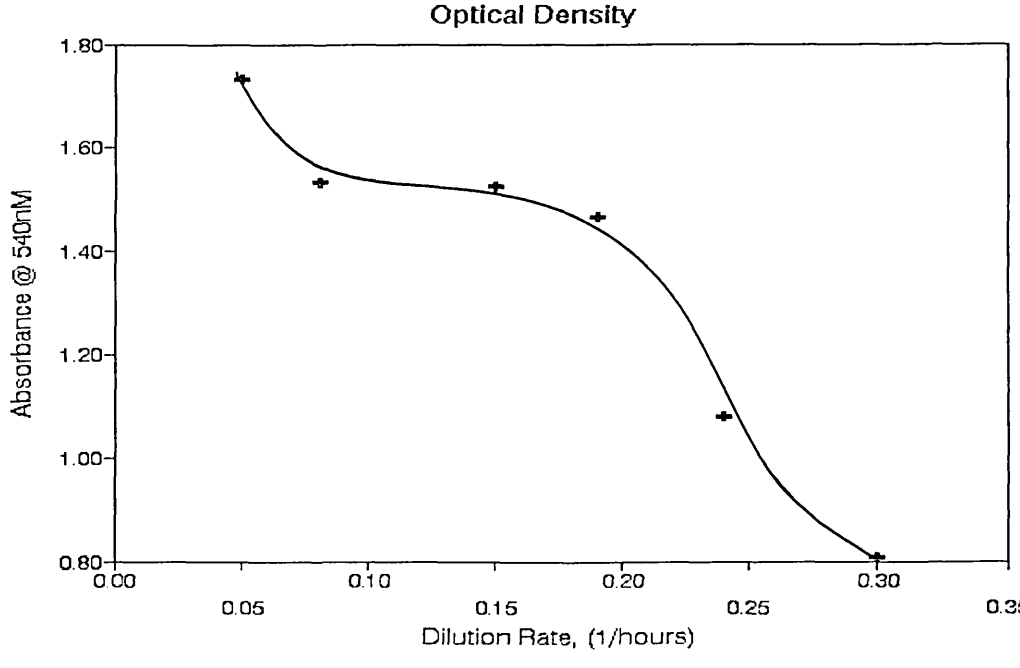


Figure 3.54: The influence of dilution rate on the dry cell weight within a chemostat operated under conditions of magnesium-limitation (90uM).

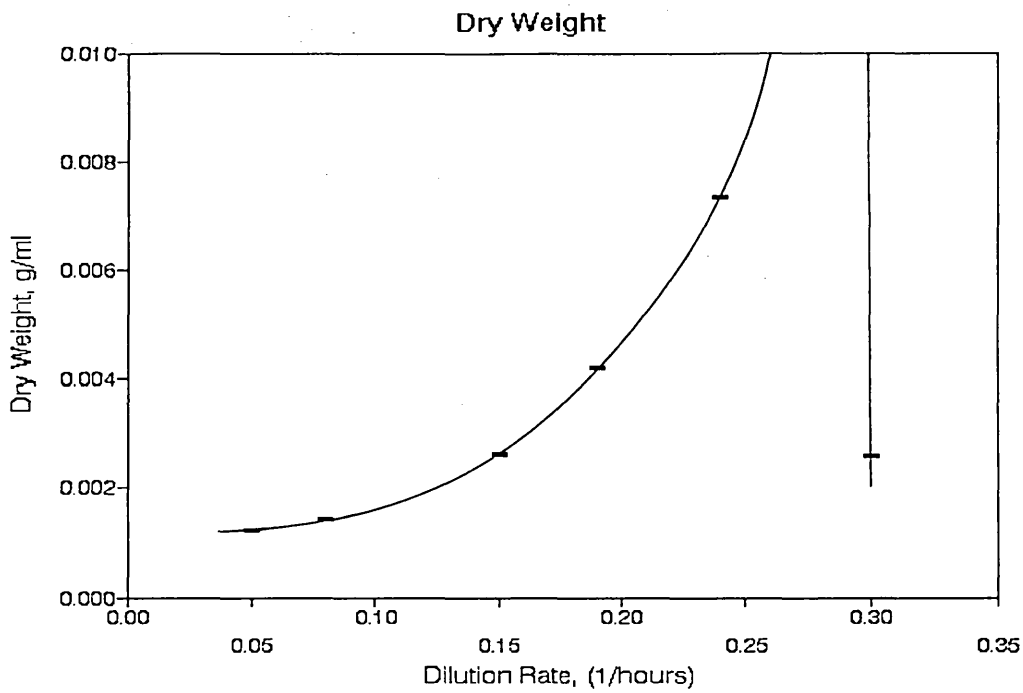


Figure 3.55: The influence of dilution rate on the mean cell volume within a chemostat operated under conditions of magnesium-limitation (90uM).

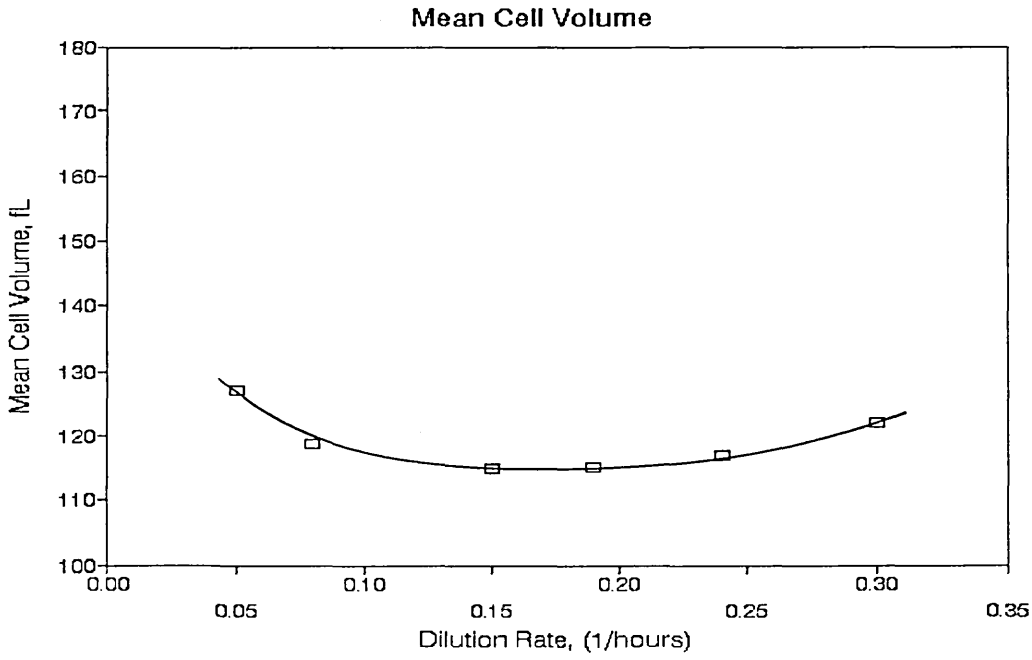


Figure 3.56: The influence of dilution rate on the ethanol concentration within a chemostat operated under conditions of magnesium-limitation (90uM).

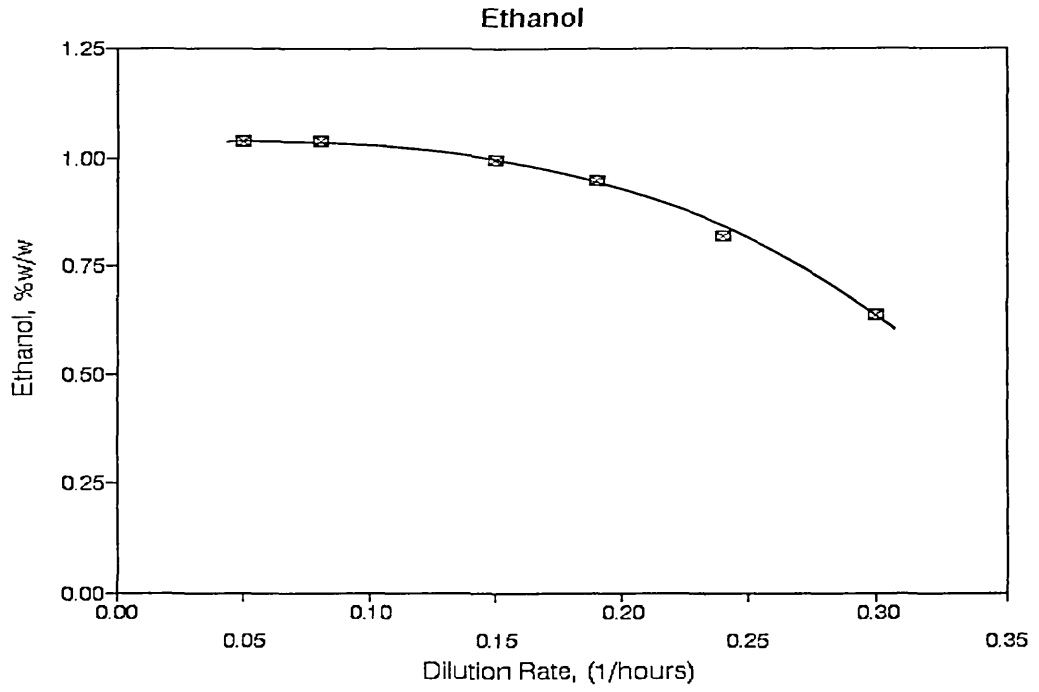


Figure 3.57: The influence of dilution rate on the glucose concentration within a chemostat operated under conditions of magnesium-limitation (90uM).

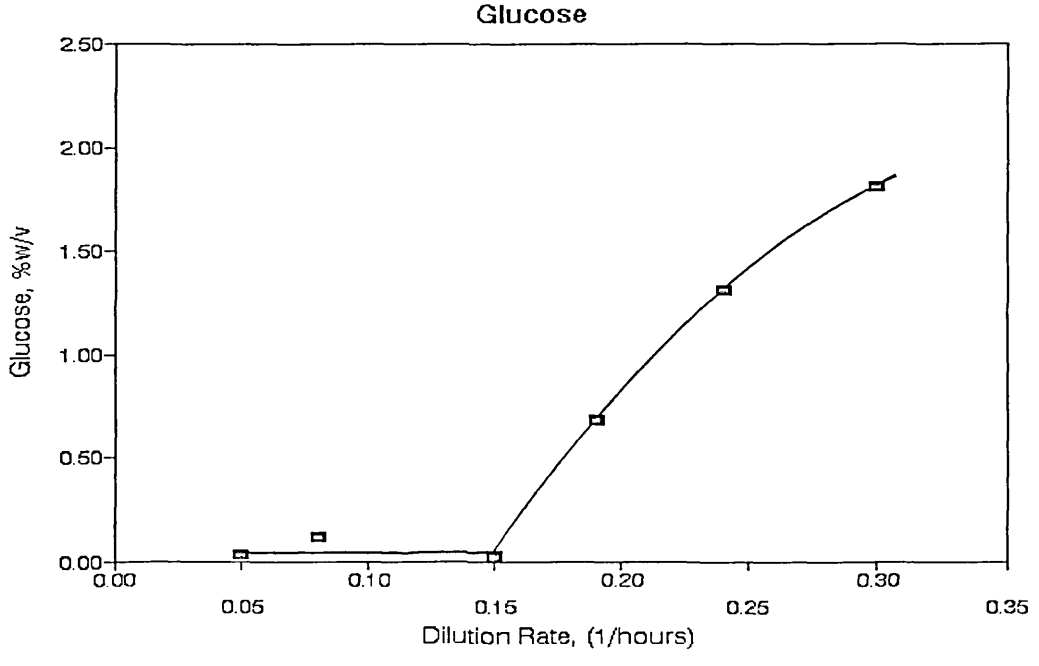


Figure 3.58: The influence of dilution rate on the magnesium concentration within a chemostat operated under conditions of magnesium-limitation (90uM).

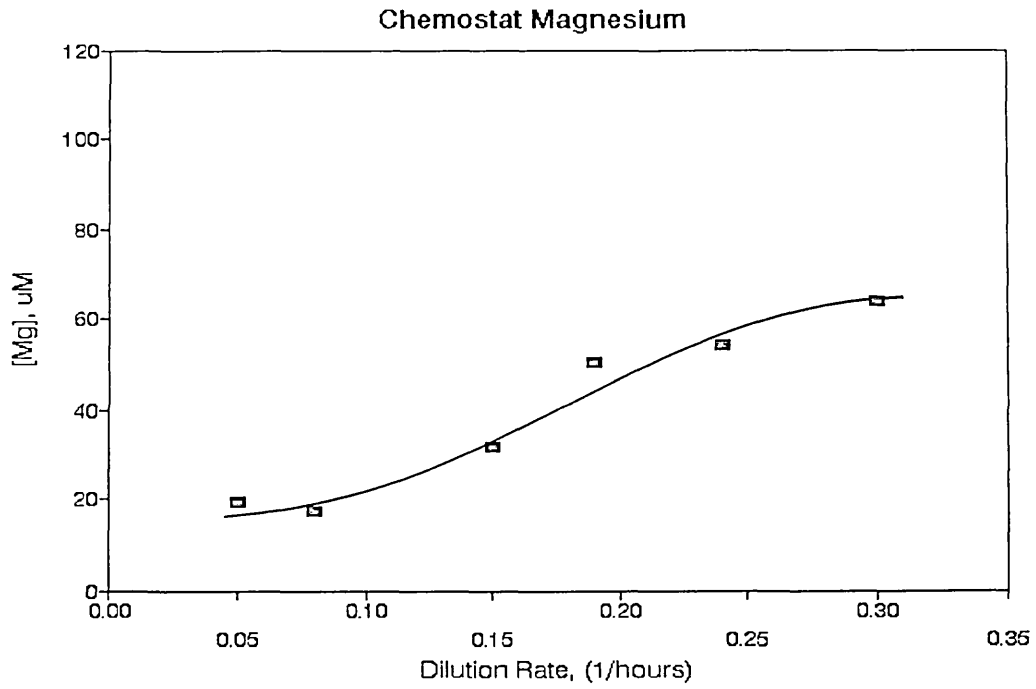


Figure 3.59: The magnesium concentration within the reservoir medium during the operation of a chemostat over a range of dilution rates.

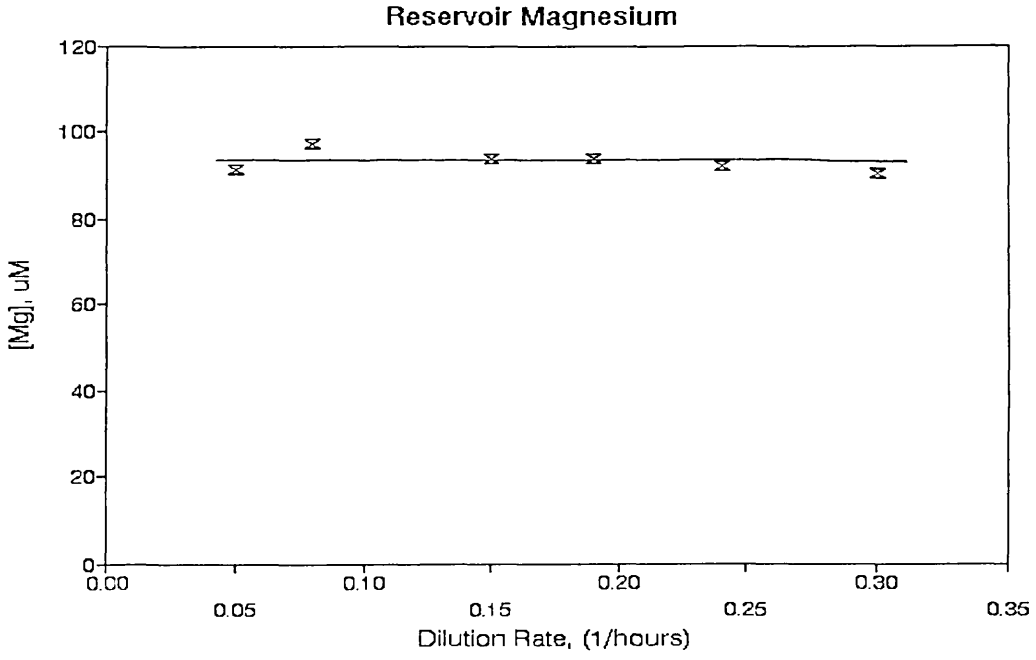


Figure 3.60: The influence of dilution rate on the cell magnesium concentration, expressed as a ratio of cell concentration, within a chemostat operated under conditions of magnesium-limitation (90uM).

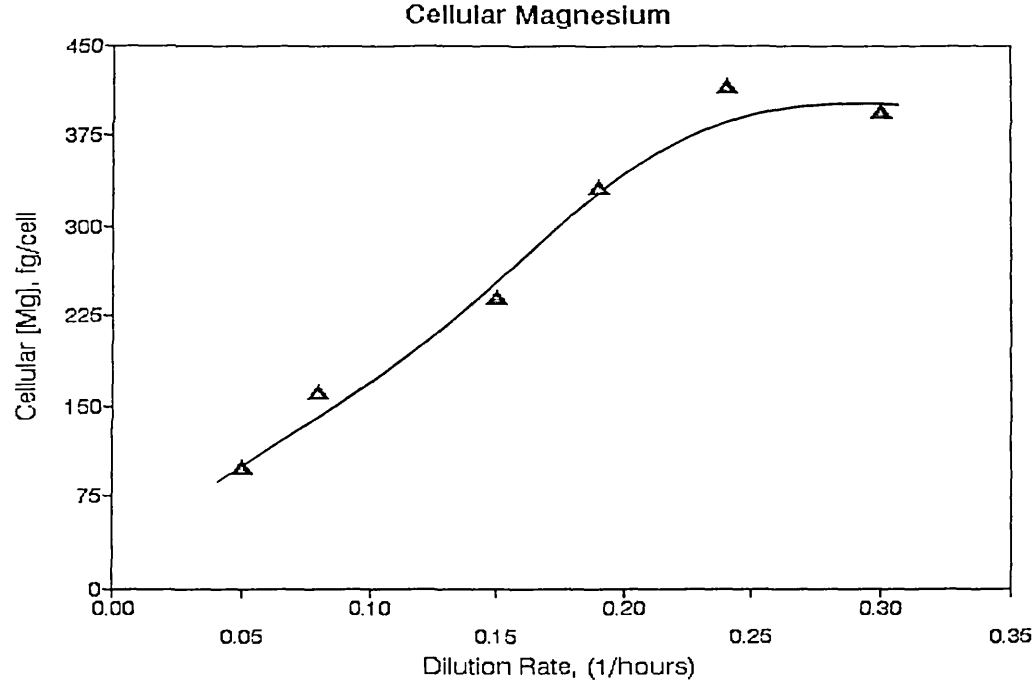


Figure 3.61: The influence of dilution rate on the cell magnesium concentration, expressed as a ratio of dry weight, within a chemostat operated under conditions of magnesium-limitation (90uM).

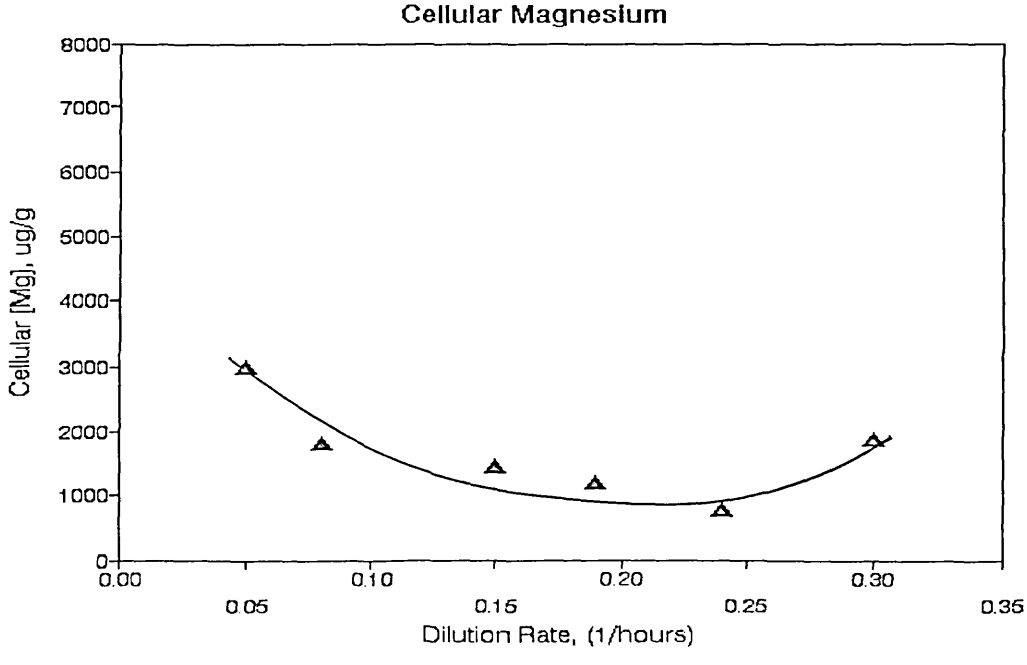


Figure 3.62: The influence of dilution rate on the oxygen uptake rate, expressed as a ratio of cell concentration, within a chemostat operated under conditions of magnesium-limitation (90uM).

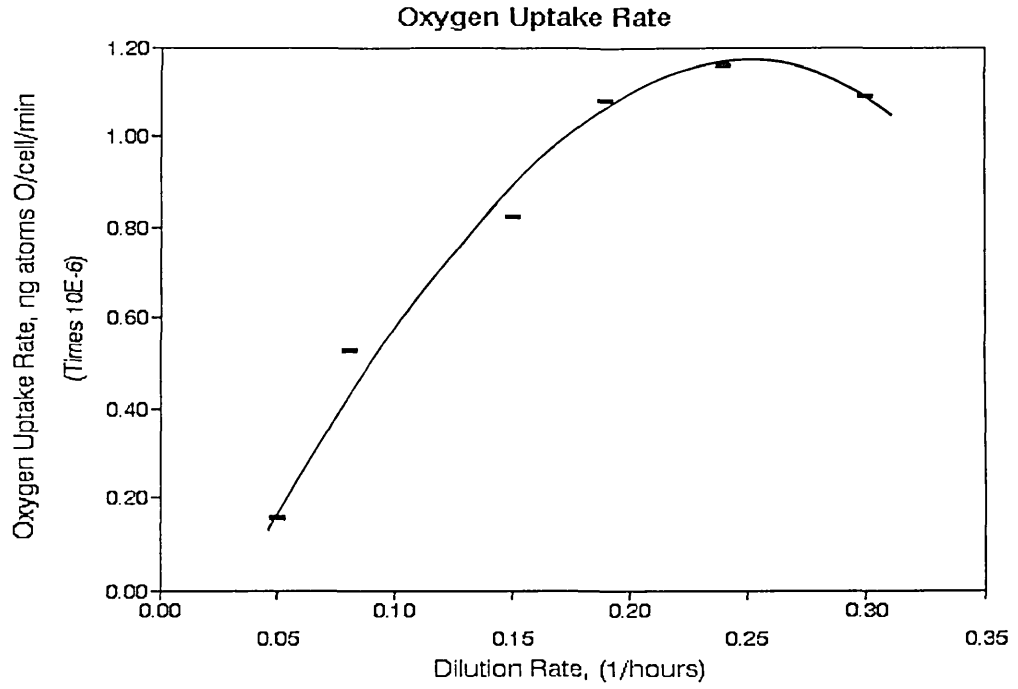


Figure 3.63: The influence of dilution rate on the oxygen uptake rate, expressed as a ratio of dry cell weight, within a chemostat operated under conditions of magnesium-limitation (90uM).

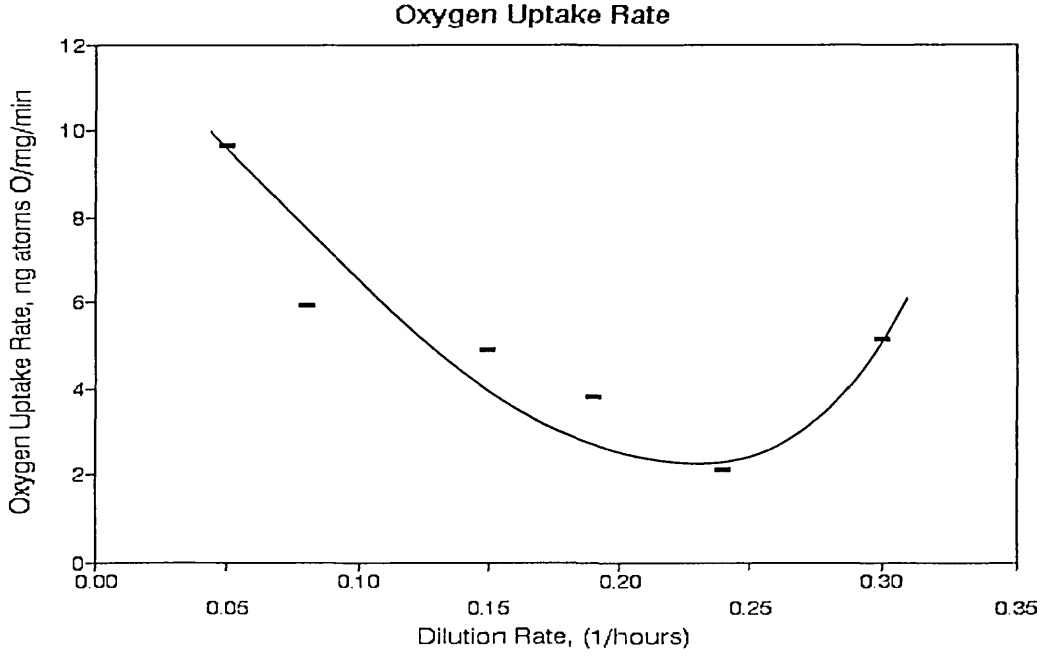


Figure 9.64: The influence of dilution rate on the pH within a chemostat operated under conditions of magnesium-limitation (90uM).

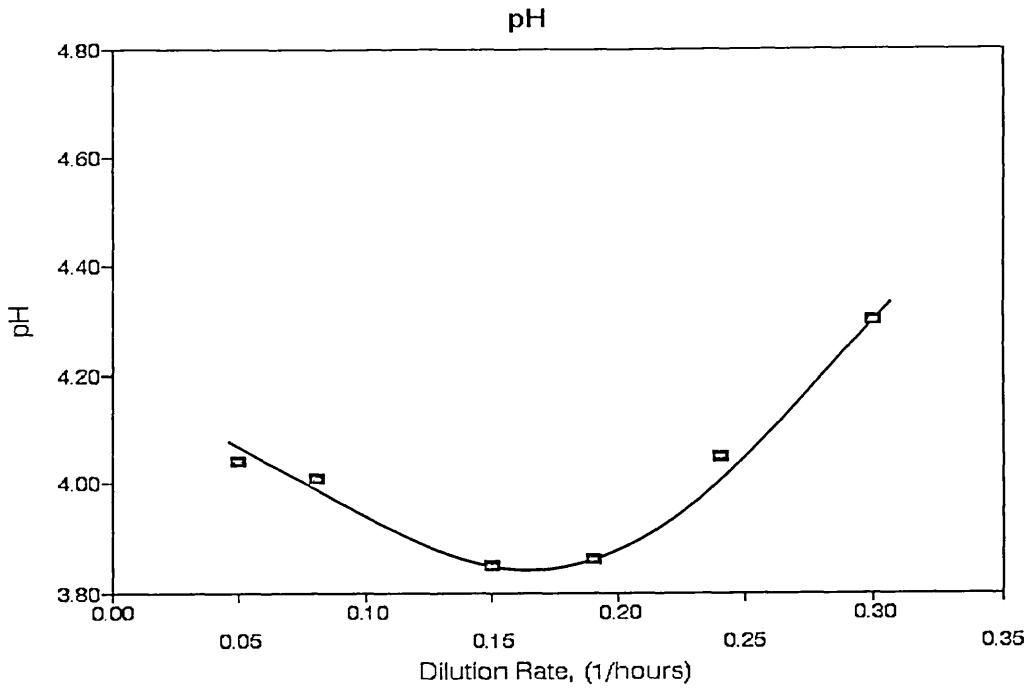


Figure 3.65: The influence of dilution rate on the biomass yield (glucose) within a chemostat operated under conditions of magnesium-limitation (90uM).

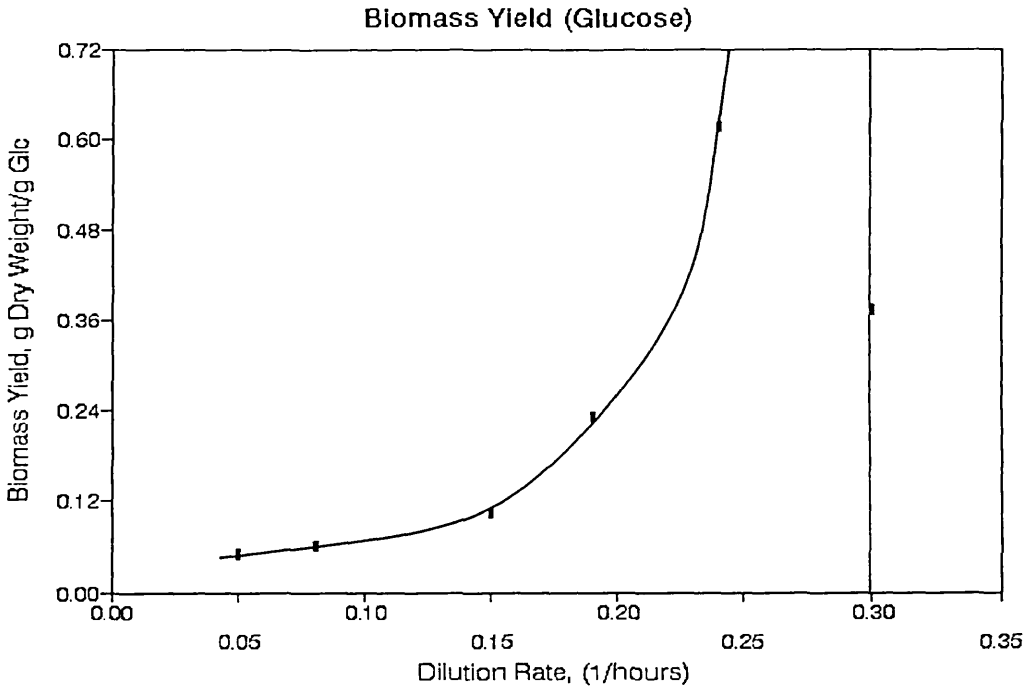


Figure 3.66: The influence of dilution rate on the ethanol yield (glucose) within a chemostat operated under conditions of magnesium-limitation (90uM).

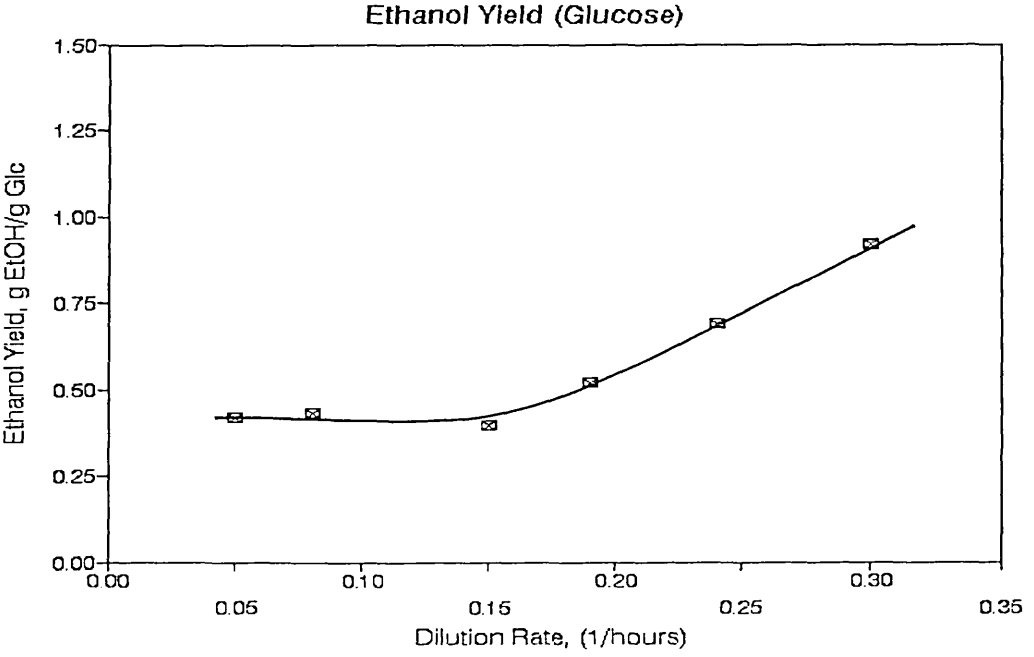


Figure 3.67: The influence of dilution rate on the biomass yield (magnesium) within a chemostat operated under conditions of magnesium-limitation (90uM).

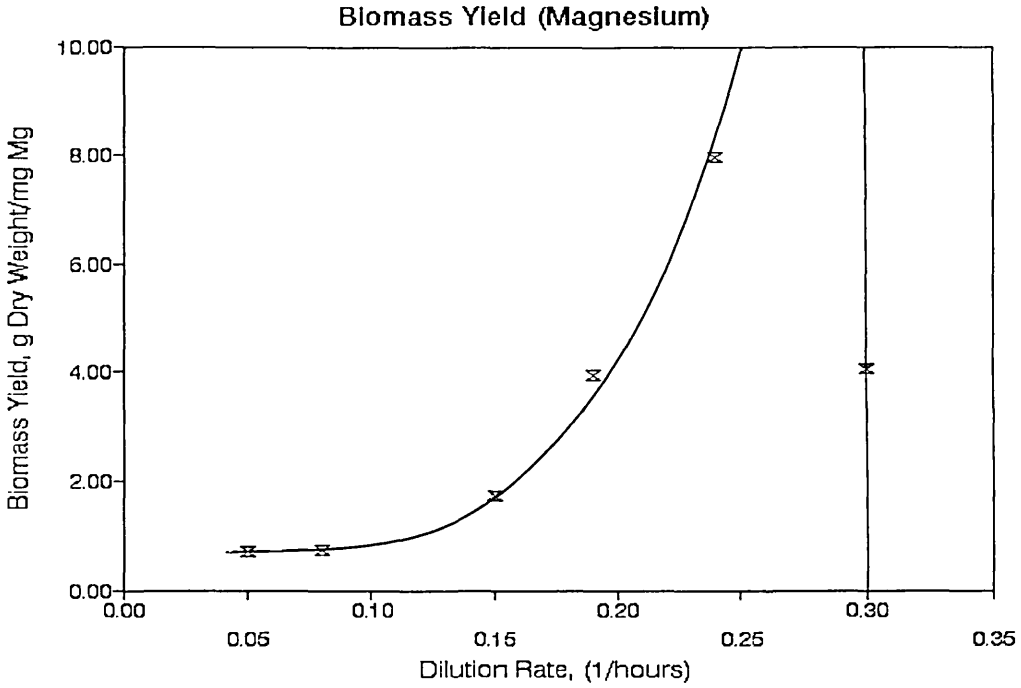


Figure 3.68: The influence of dilution rate on the ethanol yield (magnesium) within a chemostat operated under conditions of magnesium-limitation (90uM).

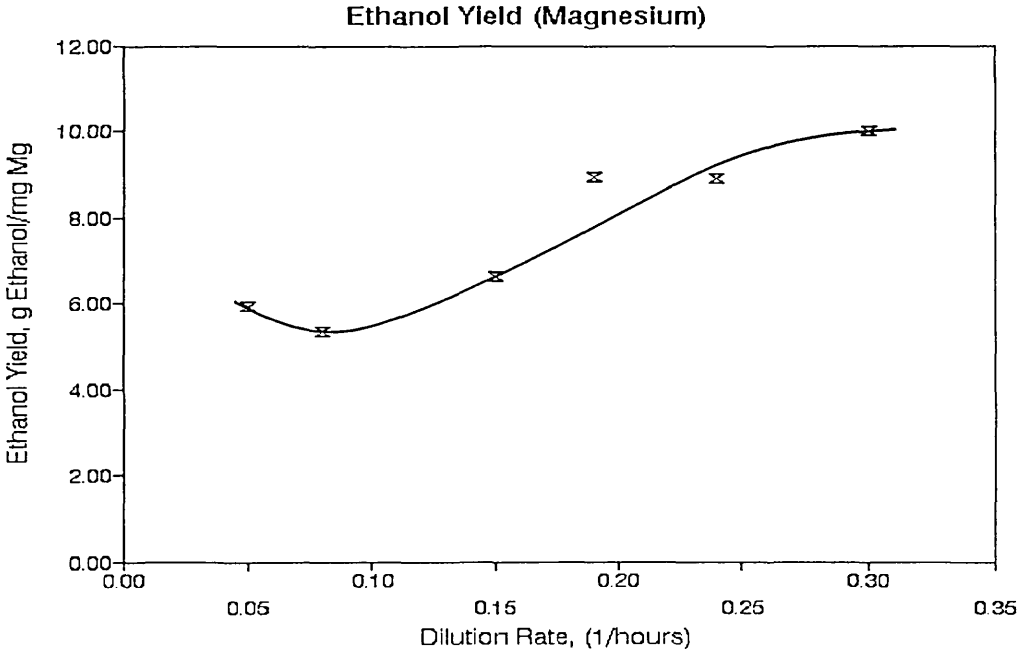


Figure 3.69: The influence of dilution rate on the ethanol production, expressed as a ratio of cell concentration, within a chemostat operated under conditions of magnesium-limitation (90uM).

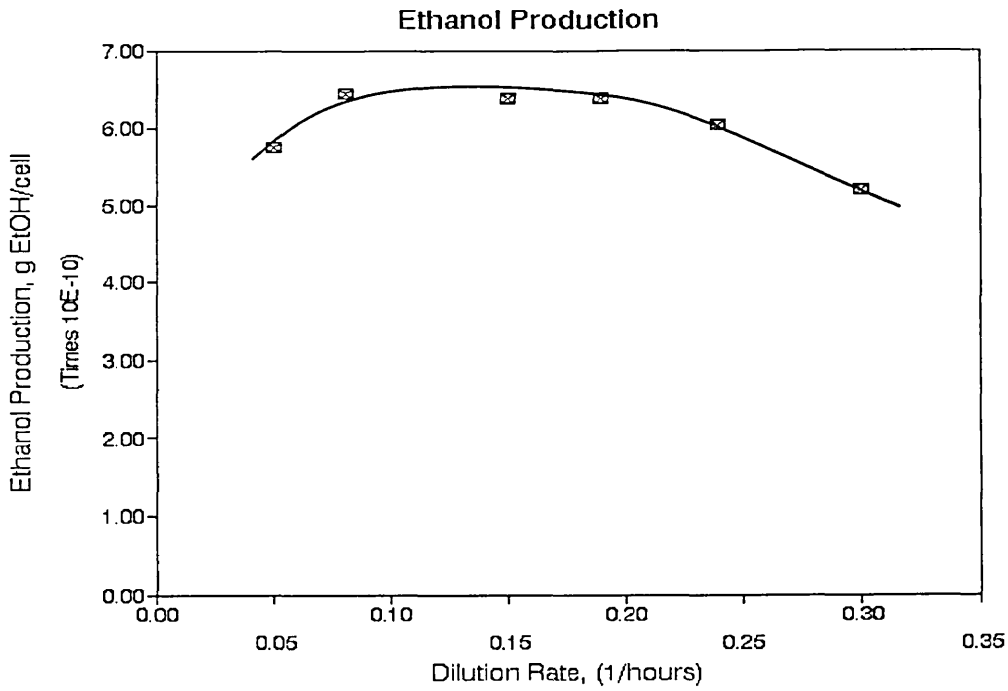


Figure 3.70: The influence of dilution rate on the ethanol production, expressed as a ratio of dry cell weight, within a chemostat operated under conditions of magnesium-limitation (90uM).

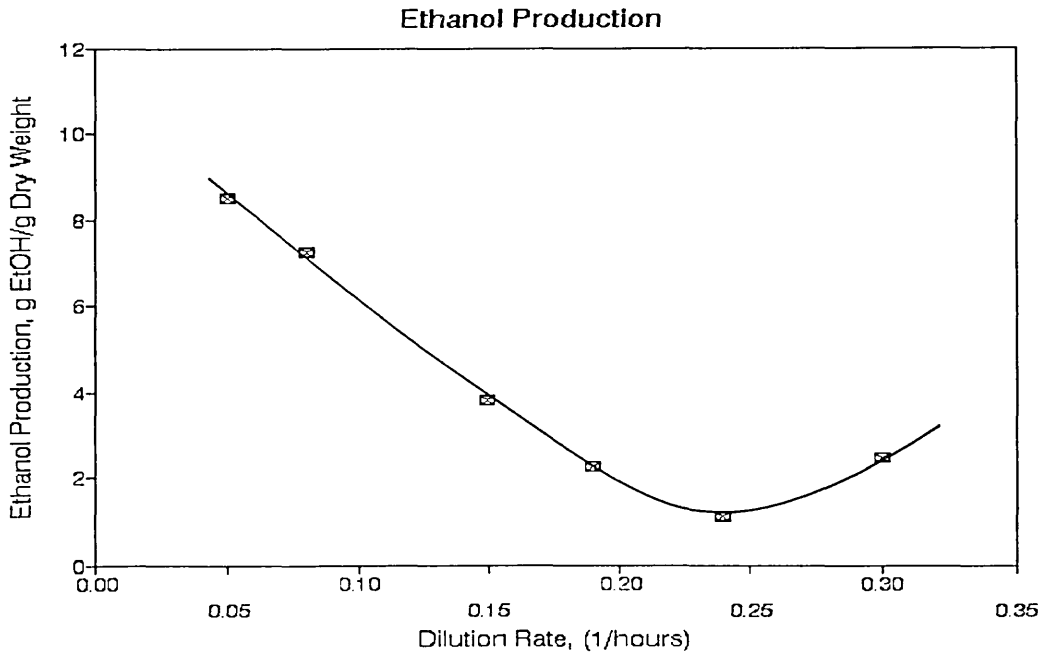


Figure 3.71: The influence of dilution rate on the glucose consumption, expressed as a ratio of cell concentration, within a chemostat operated under conditions of magnesium-limitation (90uM).

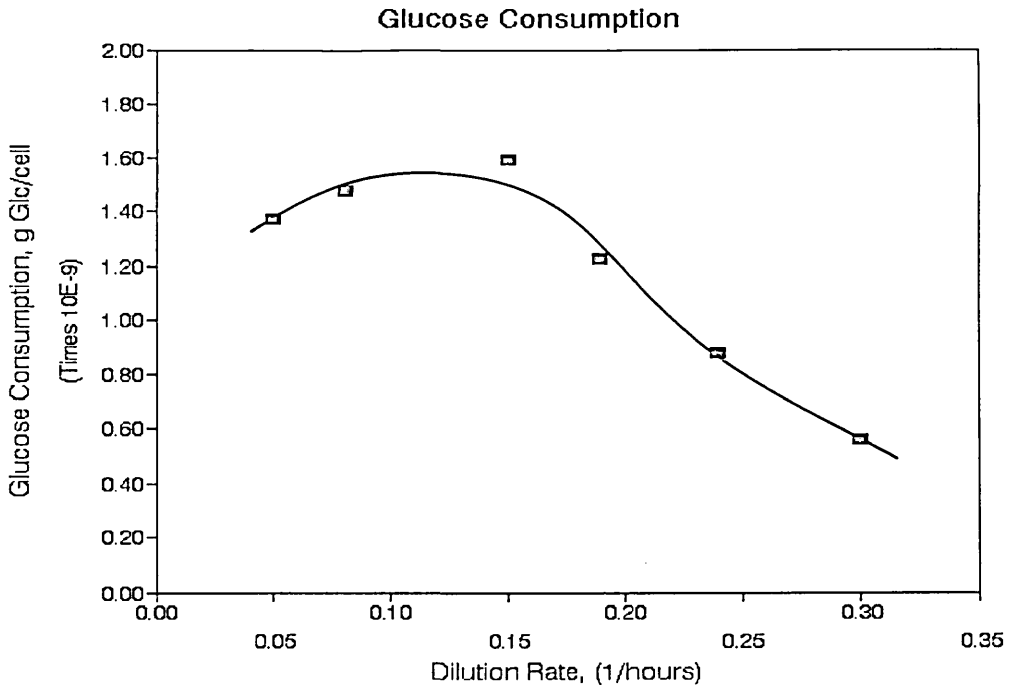
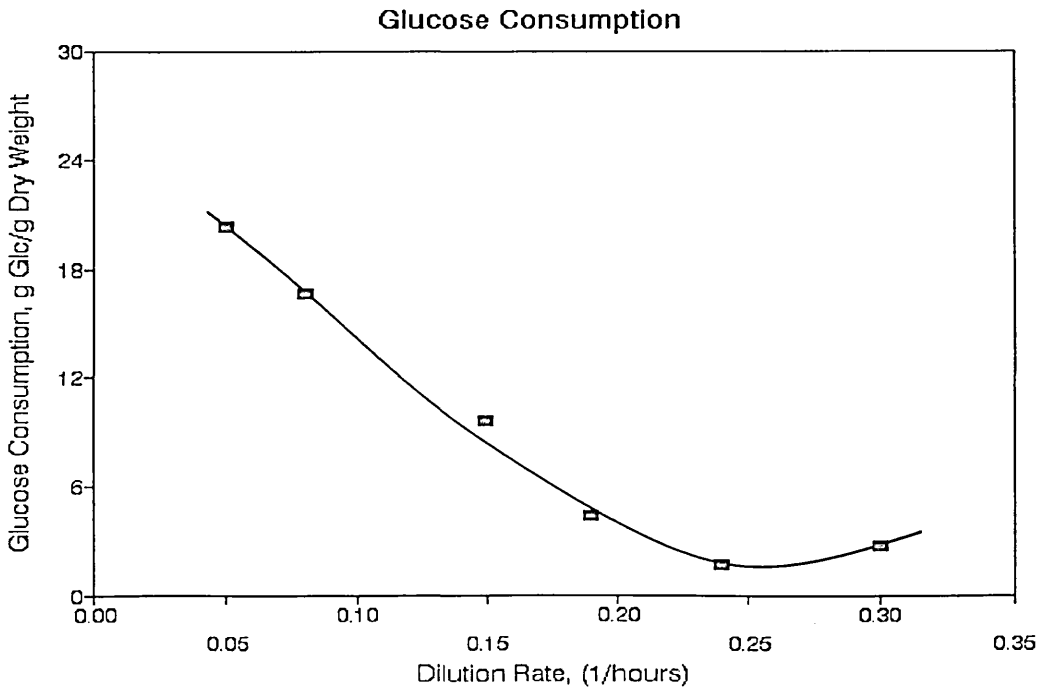


Figure 3.72: The influence of dilution rate on the glucose consumption, expressed as a ratio of dry cell weight, within a chemostat operated under conditions of magnesium-limitation (90uM).



3.2.3. The Effect of a Magnesium Pulse on the Chemostat Steady-State

3.2.3.1. Introduction

The concept of disrupting the steady-state as an experimental approach to understanding the physiological state of a cell population (Harrison and Topiwala, 1974) has already been mentioned in the overview (section 3.2.1). The recovery responses of the cell population to the perturbations are thought to reveal insights into the underlying control mechanisms of a cell's metabolism that are not usually evident during steady-state measurements. The elucidation of biochemical pathways has been achieved by measurement of the transient fluxes of the cell's constituents during the dynamic response of the culture to nutrient pulses (Bull, 1974) whilst the time delay for recovery can indicate the type of response taking place (Pye, 1969; cited by Harrison & Topiwala, 1974), e.g. the responses of substrate and biomass concentrations to flow and temperature disturbances will be fast compared to the slow response observed for changes in the inlet substrate concentration (Jefferson and Smith, 1973; cited by Harrison & Topiwala, 1974). Harrison and Topowali (1974) state that responses to a substrate pulse are similar to a stepwise increase in the dilution rate and that a lag in the response occurs if the pulse is too large.

It should be noted that the direct injection of magnesium chloride into a magnesium-limited (62μM) chemostat culture of *K. pneumoniae* was regarded by Buurman, Boiardi, Teixeira de Mattos, & Neijssel (1990) as being a "pulse-experiment" whereas this study has specified direct injections of magnesium into the chemostat as being 'spikes' and increases in the magnesium concentration within the feed medium as being 'pulses'.

In view of the data presented in section 3.2.2, a slow increase in the available magnesium should effectively result in a transition from a predominance of fermentation to respiration followed by a reversal of this effect as the pulse fades away. Effectively, the cells will be responding on a global level through replicative behaviour

and metabolic behaviour. Changes in intermediary metabolism should allow an increased opportunity for the cells to grow in an assimilatory manner whilst changes in replicatory behaviour should allow an increased opportunity for the population to grow.

3.2.3.2. Method

The chemostat set-up has been detailed in section 2.2.1; the medium preparation in section 2.2.2; the inoculum preparation in section 2.2.3; and the analyses of samples in section 2.3.

Continuous culture was initiated as described in section 2.2.3 with the exception that the dilution rate was set immediately at $0.15hrs^{-1}$ and the chemostat allowed to equilibrate for four fermenter volumes. The chemostat was then sampled, allowed to recover, sampled a second time and again a re-equilibration permitted before a third sample was removed from the chemostat whereupon the system was allowed to establish a steady-state over four fermenter volumes.

Once steady-state had been achieved, the pump from the main reservoir to the mixing vessel (Figure 2.1) was switched to its maximum pumping rate and the mixing vessel allowed to fill to a previously calibrated mark; set at 1.9 litres. The pump was then turned off and 2mls of the magnesium stock solution were added to the mixing vessel. This effectively increased the feed concentration of magnesium from 50μM to 140μM and supplied the chemostat for 16hrs before the pump had to be turned back on at its normal rate; i.e. equal to the pump between the mixing vessel and the chemostat; to maintain a flow of nutrients. The procedure was repeated for a pulse of 300μM.

Normal sampling according to section 2.2.3 could not be followed due to the disruption to the chemostat's operating volume and therefore one set of samples was taken directly from the chemostat outlet pipe before entry to the waste vessel. This technique had limitations regarding the rate at which samples could be accumulated (especially for dry weight determination and hence the cellular magnesium concentrations); and therefore only one sample was taken by this method with the

second, duplicate sample being extracted from the chemostat as described in section 2.2.3. The disruption was held to be tolerable.

3.2.3.3. Results and Discussion

The Effect of a 140μM Magnesium Pulse on the Chemostat Steady-State

The results detail several directly measured and derived fermentative and respirative aspects of the continuous, magnesium-limited growth of a cell population of *S. cerevisiae* responding to a pulse of magnesium generated from within the mixing vessel.

Changes in the measured parameters (cell concentration, medium absorbance, cell dry weight, mean cell volume, medium ethanol, glucose, and magnesium, cellular magnesium levels, oxygen uptake rate, medium pH and oxygen levels) as a function of the magnesium-controlled growth rate are shown in Figures 3.73, 3.74, 3.75, 3.76, 3.77, 3.78, 3.79, 3.80, 3.81, 3.82, 3.83, 3.84, 3.85, and 3.86 respectively. The effect of growth rate on the derived variables of biomass and ethanol yields (glucose and magnesium), ethanol production (cell and dry weight) and glucose uptakes (cell and dry weight) are shown in Figures 3.87 and 3.88, 3.89 and 3.90, 3.91, 3.92, 3.93, and 3.94 respectively. The initiation of the pulse is indicated by the dotted line at zero hours with the steady-state parameter values being given prior to this zero time. The results for the magnesium pulse of 300μM are not presented here as the trends are identical to the trends appearing for a 140μM magnesium pulse.

The results for the cell concentration (Figure 3.73), optical density (Figure 3.74), and dry weight (Figure 3.75) all show a smooth, progressive increase in the cell population as the magnesium concentration within the chemostat (Figure 3.79) is raised due to the extra magnesium present within the mixing vessel (Figure 3.80). The maxima for these curves all appear at 16hrs as does the end of the pulse. The curve for the mean cell volume (Figure 3.76) is complementary to the cell concentration in that it indicates an increase in cell division with the associated production of many smaller

daughter cells. These daughter cells then appear to grow in size as indicated by the recovery of the cell volume as the pulse fades away. The increase in cell numbers superficially accounts for the increase in dry weight inferring that the cells may become smaller but do not become more dense.

The ethanol concentration (Figure 3.77) within the chemostat rises concomitantly with the increase in cell numbers and dry weight, also reaching its highest value at 16hrs. However, as can be seen from Figures 3.91 and 3.92, the ethanol production is not affected by these changes but rather it remains constant during the expansion of the population and therefore the rise in ethanol is accounted for by the simple increase in the cell population. Interestingly, it is only when the pulse is over and the cell population is being washed out of the chemostat, by the progressive return to magnesium-limitation, that the fermentative metabolism of the cells changes, as indicated by the rise in ethanol production after the 16 hour mark.

Similarly, during the expansion of the cell population, the biomass yield from glucose (Figure 3.87) is not affected, again inferring that the cells are not altering their metabolism to utilise the glucose on a fermentative basis but rather the decrease in the chemostat glucose concentration (Figure 3.78) as the pulse progresses is directly accounted for by the increase in biomass. Again however, there does appear to be a change in cell metabolism after the 16 hour mark as the biomass yield decreases.

The pattern is repeated for the glucose consumption (Figures 3.93 and 3.94) where the glucose consumption remains level for the duration of the pulse but begins to rise after 16hrs.

The pattern is not repeated for the medium oxygen levels (Figure 3.86) where a decrease is observed as the pulse intensifies but instead of this being accounted for by the changes in cell concentration or biomass it can be seen from Figures 3.83 and 3.84

that the oxygen uptake rate per cell or per milligram of dry weight does not remain constant but instead decreases. This would infer that there is a change in the metabolism of the cells towards a less respirative state without any compensatory rise in fermentative metabolism (as argued above).

Figures 3.81 and 3.82 show that during the time when the fermentative metabolism of the cell is remaining constant, the cellular magnesium levels are increasing; but when the pulse fades, the fermentative metabolism increases and the cellular magnesium concentration falls. This is in keeping with the results of section 3.2.2.3: magnesium limitation at 50μM where ethanol production was greatest when the cellular magnesium levels were lowest.

Overall, it would appear that the magnesium pulse lifts all limitations in cell growth and allows an expansion of the cell population to occur without affecting the fermentative metabolism of the cells although a decrease in the relative contribution of respiration to the respiro-fermentative state is inferred by the decrease in the oxygen uptake rate. When the pulse fades away, the limitations on cell growth return and the population contracts and it is then that changes in the cells fermentative metabolism appear.

The oxygen uptake rate of the cells decreases during the pulse but the levels of oxygen within the medium also decrease. Thus, the overall increase in cell biomass causes a depletion of oxygen from the medium, possibly for the synthesis of cell-membrane components as mentioned in section 1.2.3.2., but the cells on an individual basis utilize less oxygen. This could be accounted for by inferring that the respiratory capacity of the cells is not increasing with the increase in biomass but is instead remaining at a constant level as hypothesized from the results of section 3.2.2.3: magnesium limitation at 90μM. However, it is impossible to differentiate between the oxygen used for structural purposes and that used for respiratory activities using the data presented here.

Figure 3.73: The influence of a 140uM magnesium-pulse on the steady-state cell concentration within a chemostat held at a dilution rate of 0.15/hrs originally derived from a magnesium-limitation of 50uM.

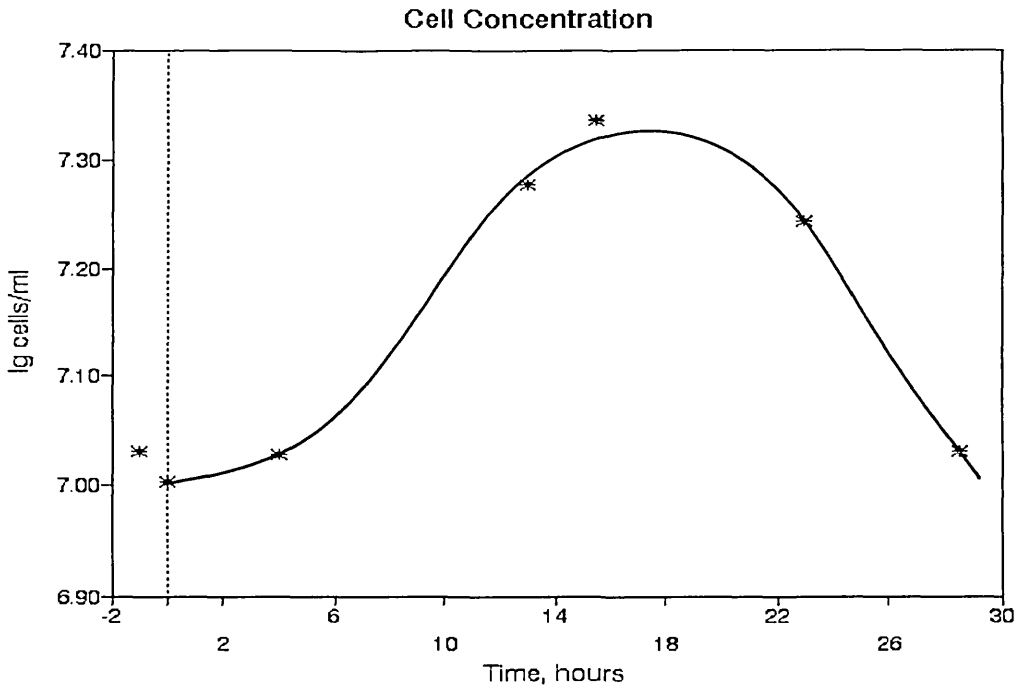


Figure 3.74: The influence of a 140uM magnesium-pulse on the steady-state optical density within a chemostat held at a dilution rate of 0.15/hrs originally derived from a magnesium-limitation of 50uM.

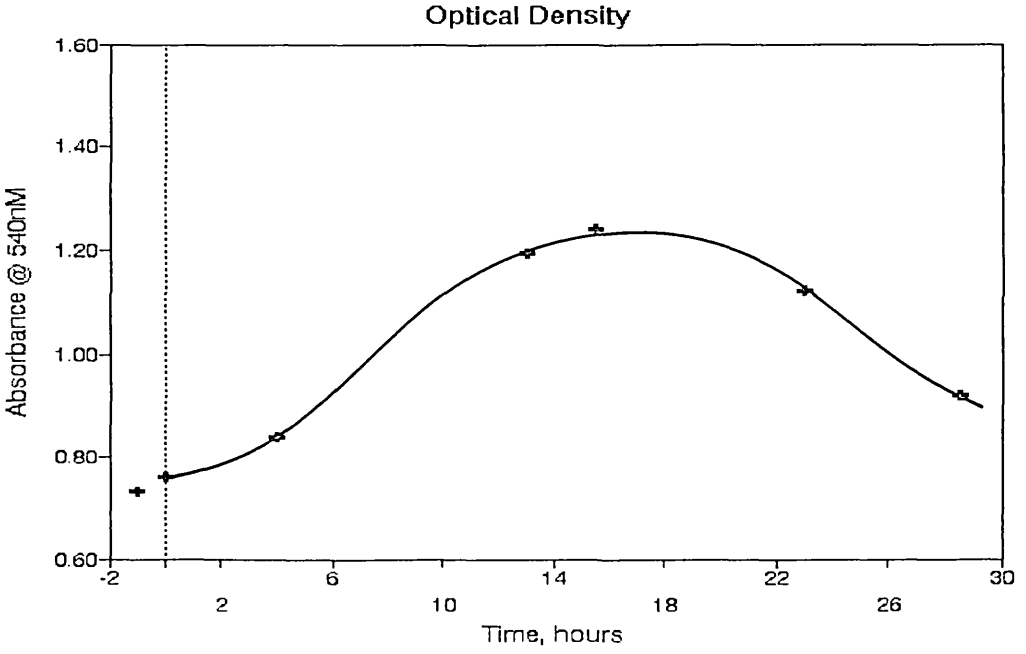


Figure 3.75: The influence of a 140uM magnesium-pulse on the steady-state cell dry weight within a chemostat held at a dilution rate of 0.15/hrs originally derived from a magnesium-limitation of 50uM.

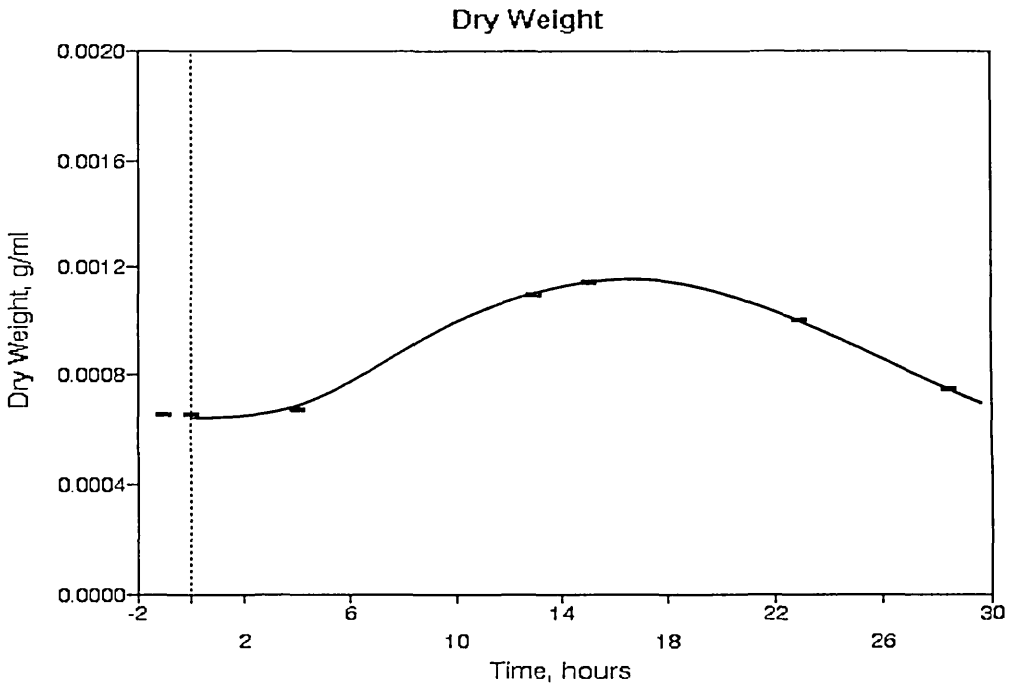


Figure 3.76: The influence of a 140uM magnesium-pulse on the steady-state mean cell volume within a chemostat held at a dilution rate of 0.15/hrs originally derived from a magnesium-limitation of 50uM.

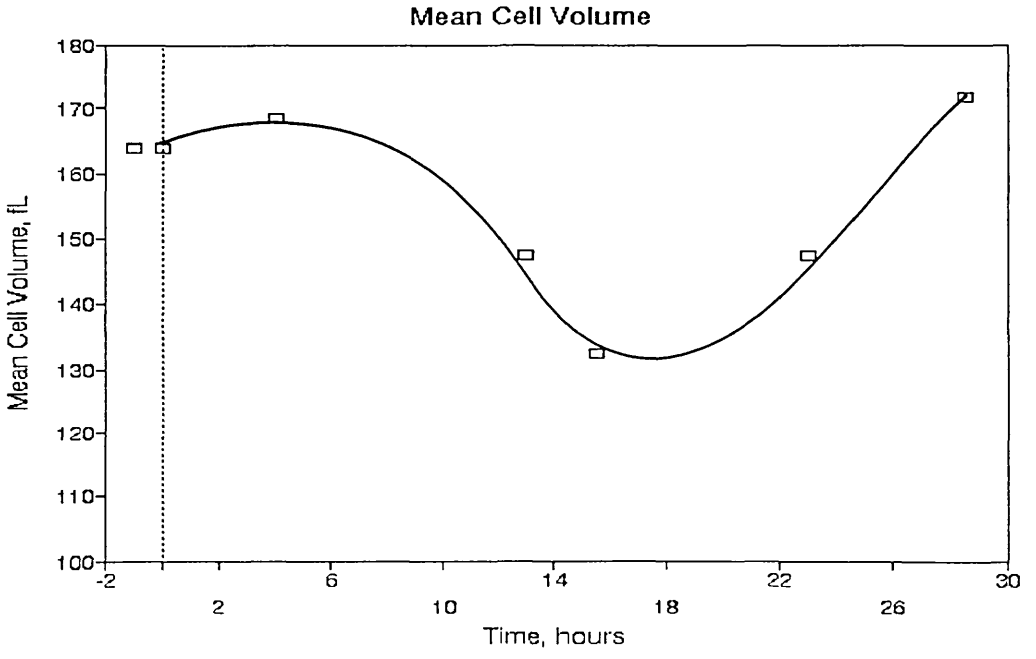


Figure 3.77: The influence of a 140uM magnesium-pulse on the steady-state ethanol concentration within a chemostat held at a dilution rate of 0.15/hrs originally derived from a magnesium-limitation of 50uM.

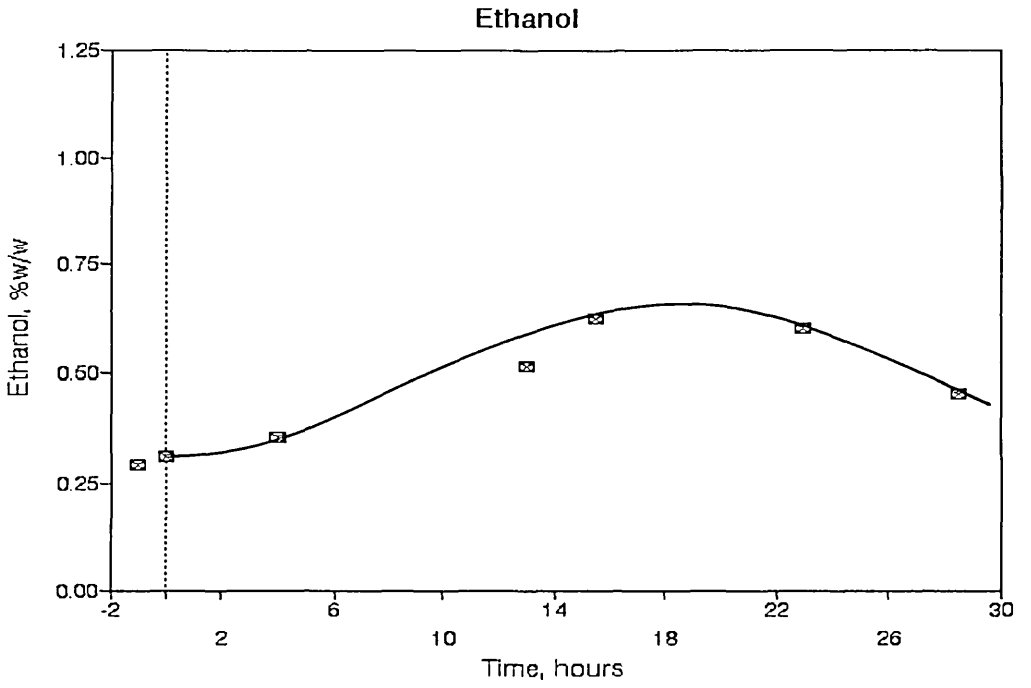


Figure 3.78: The influence of a 140uM magnesium-pulse on the steady-state glucose concentration within a chemostat held at a dilution rate of 0.15/hrs originally derived from a magnesium-limitation of 50uM.

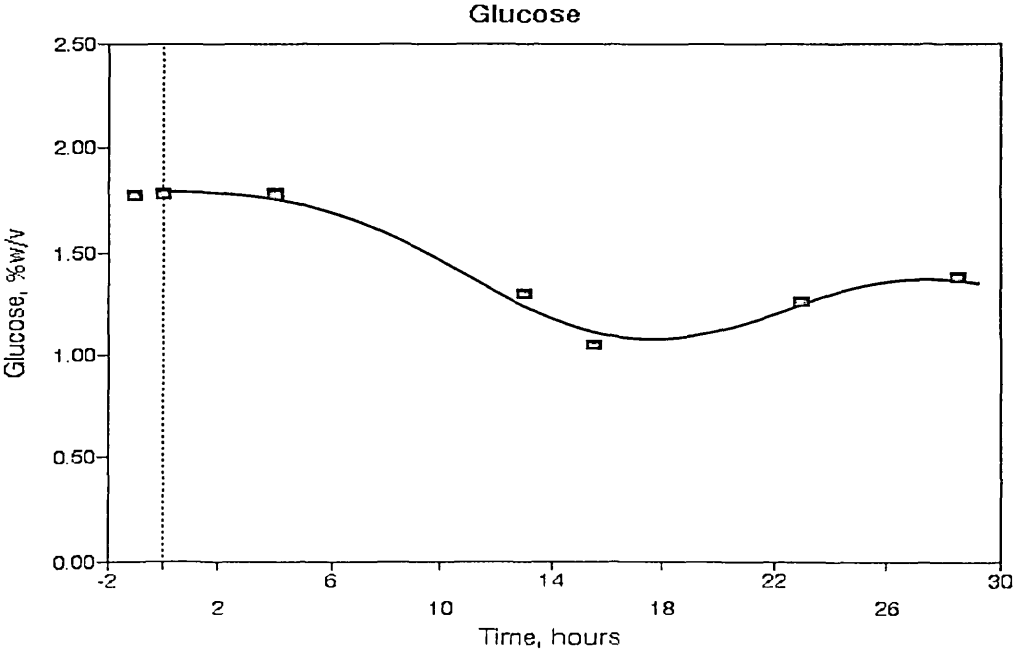


Figure 3.79: The influence of a 140uM magnesium-pulse on the steady-state magnesium concentration within a chemostat held at a dilution rate of 0.15/hrs originally derived from a magnesium-limitation of 50uM.

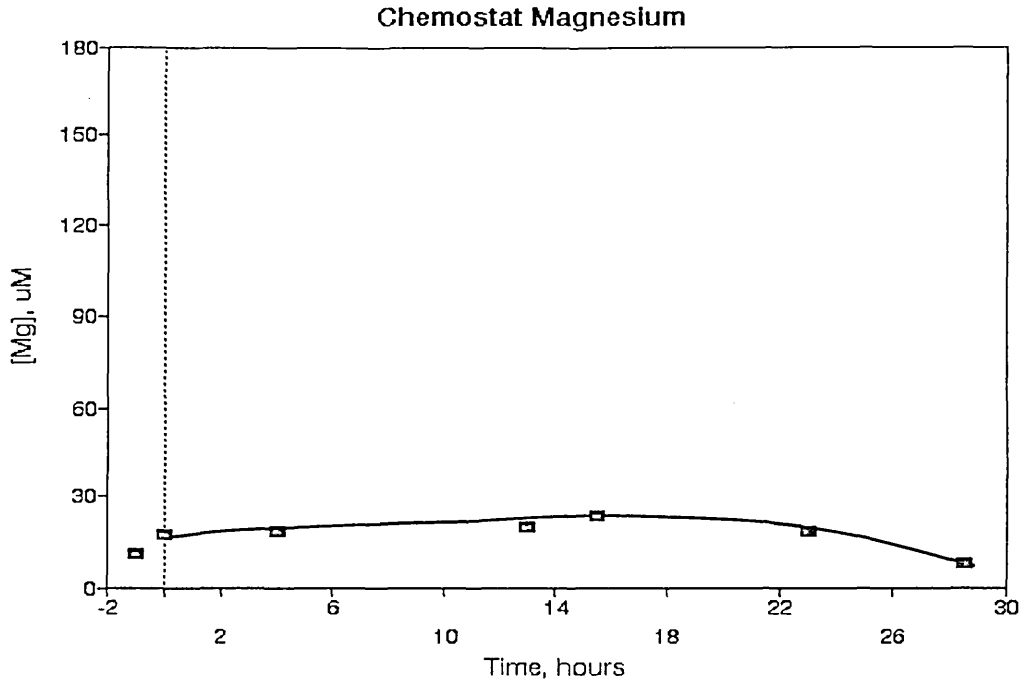


Figure 3.80: The magnesium concentration within the reservoir medium before and after the addition of magnesium required to generate the magnesium-pulse within the chemostat.

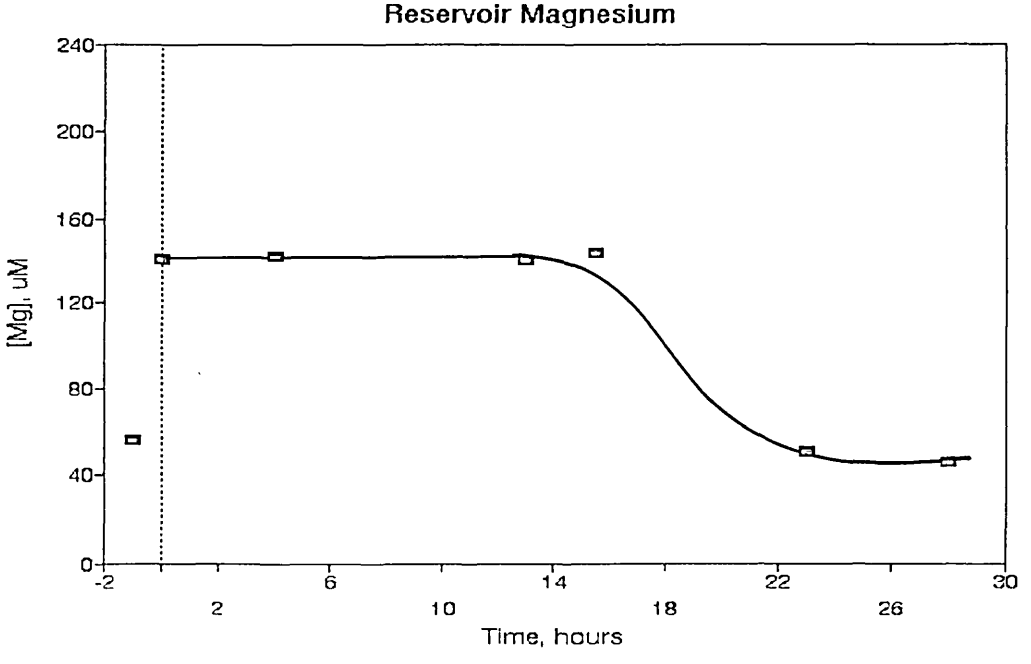


Figure 3.81: The influence of a 140uM magnesium-pulse on the steady-state cell magnesium concentration, expressed as a ratio of cell concentration, within a chemostat held at a dilution rate of 0.15/hrs originally derived from a magnesium-limitation of 50uM.

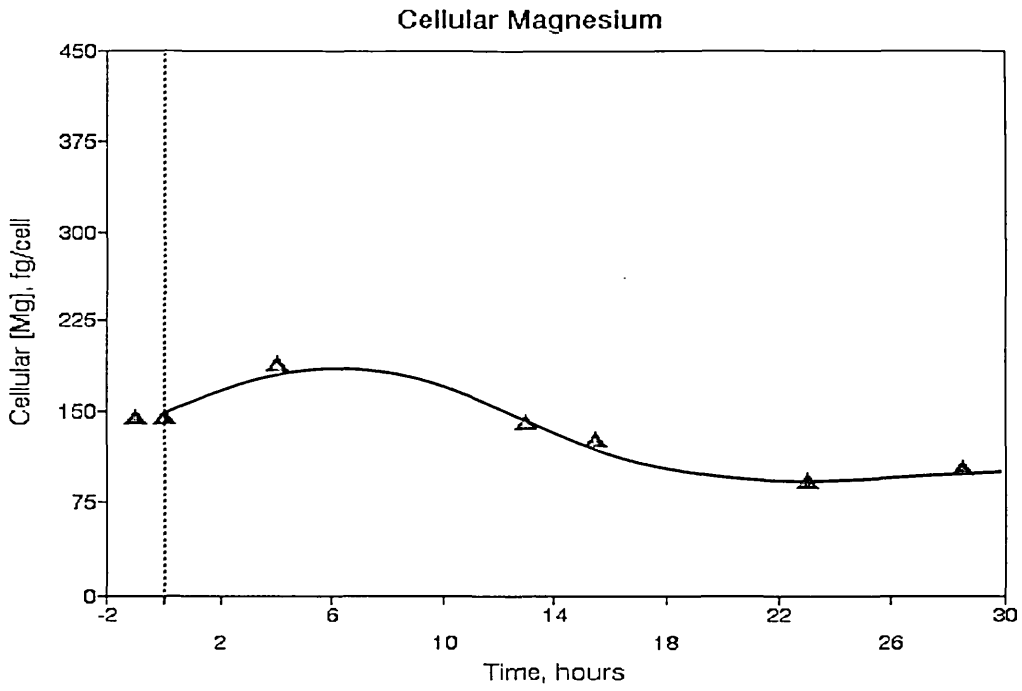


Figure 3.82: The influence of a 140uM magnesium-pulse on the steady-state cell magnesium concentration, expressed as a ratio of dry weight, within a chemostat held at a dilution rate of 0.15/hrs originally derived from a magnesium-limitation of 50uM.

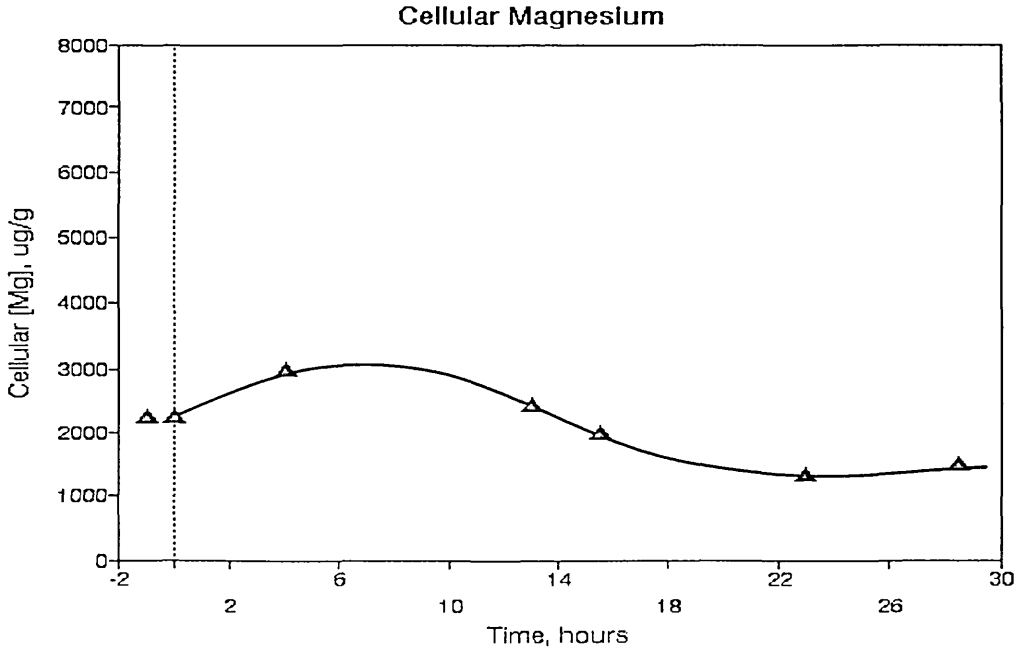


Figure 3.83: The influence of a 140uM magnesium-pulse on the steady-state oxygen uptake rate, expressed as a ratio of cell concentration, within a chemostat held at a dilution rate of 0.15/hrs originally derived from a magnesium-limitation of 50uM.

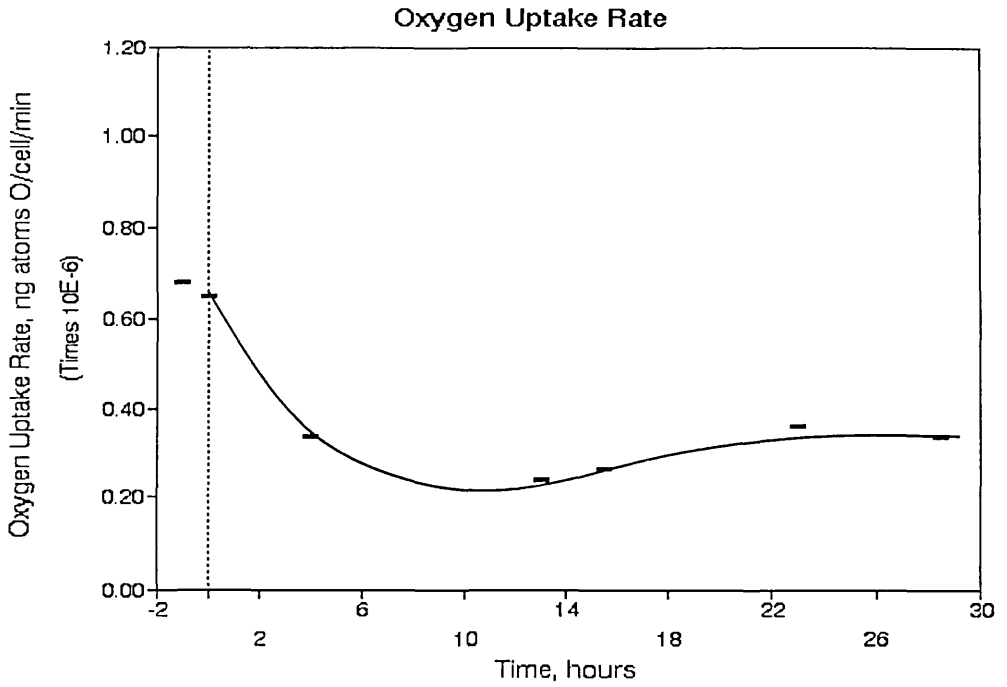


Figure 3.84: The influence of a 140uM magnesium-pulse on the steady-state oxygen uptake rate, expressed as a ratio of dry weight, within a chemostat held at a dilution rate of 0.15/hrs originally derived from a magnesium-limitation of 50uM.

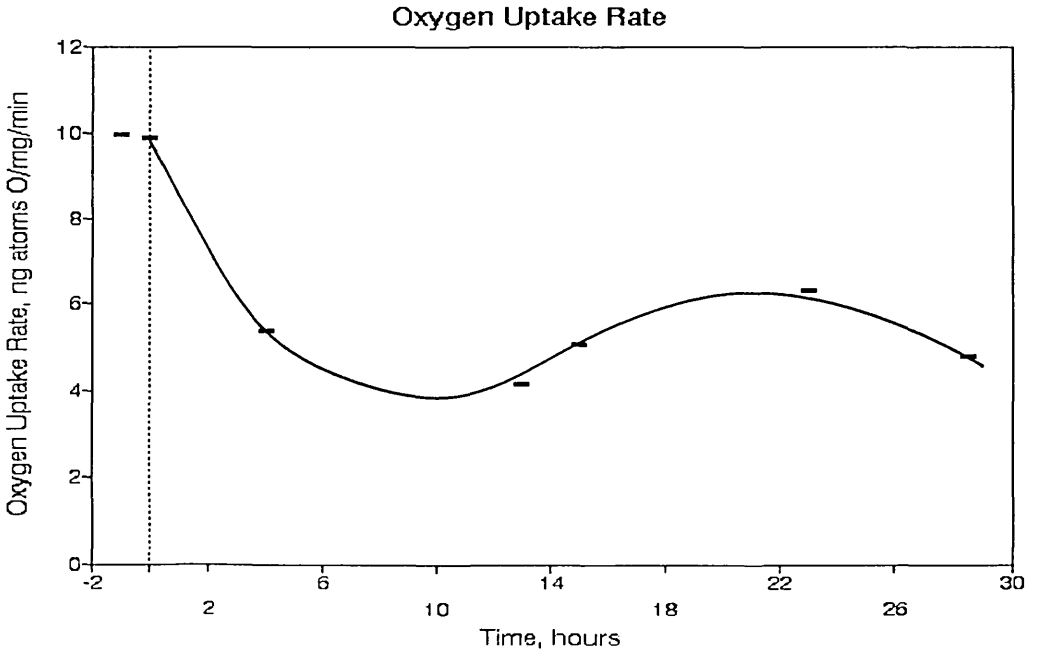


Figure 3.85: The influence of a 140uM magnesium-pulse on the steady-state pH within a chemostat held at a dilution rate of 0.15/hrs originally derived from a magnesium-limitation of 50uM.

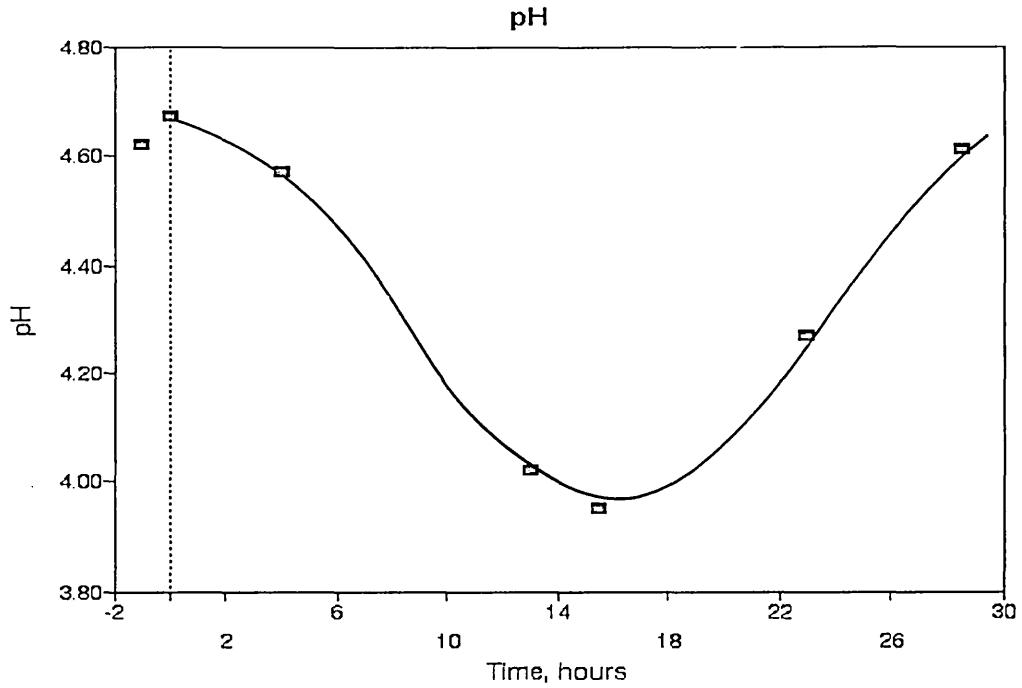


Figure 3.86: The influence of a 140uM magnesium-pulse on the steady-state oxygen levels within a chemostat held at a dilution rate of 0.15/hrs originally derived from a magnesium-limitation of 50uM.

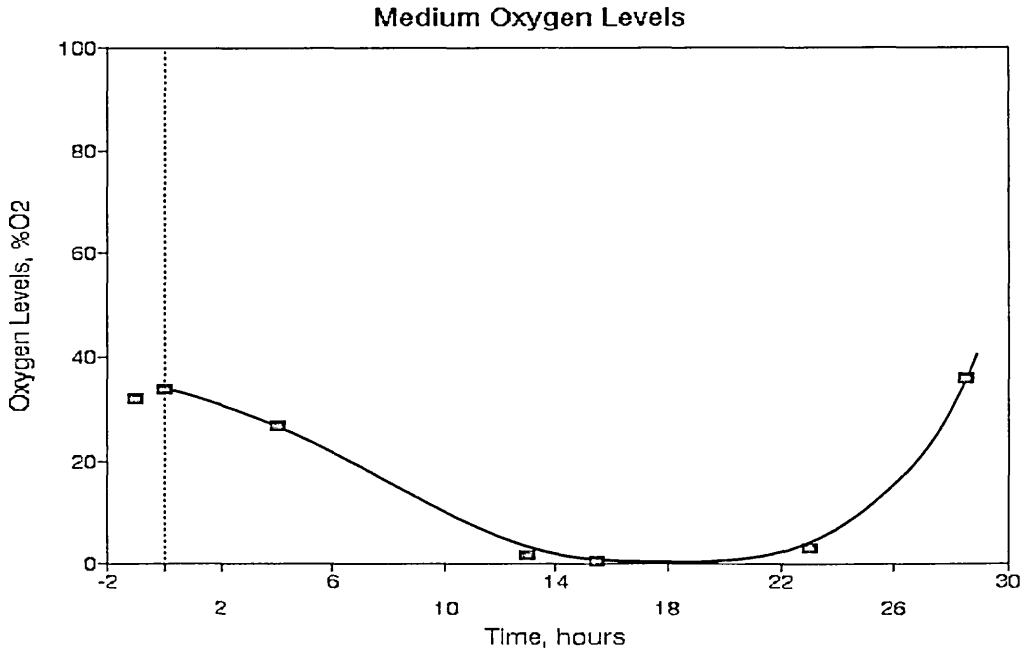


Figure 3.87: The influence of a 140uM magnesium-pulse on the steady-state biomass yield (glucose) within a chemostat held at a dilution rate of 0.15/hrs originally derived from a magnesium-limitation of 50uM.

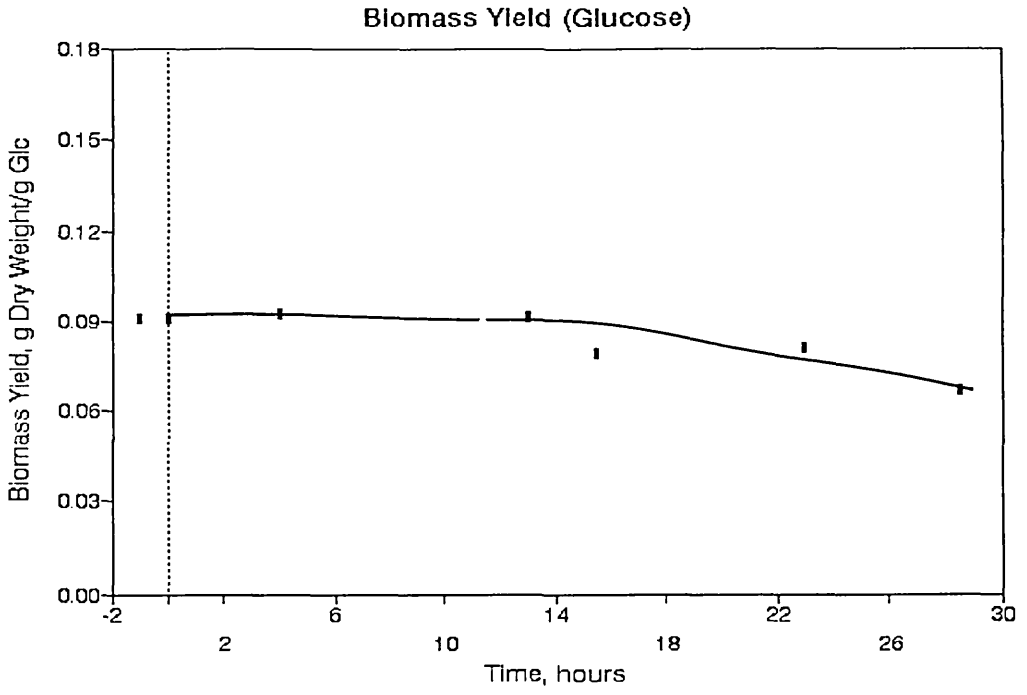


Figure 3.88: The influence of a 140uM magnesium-pulse on the steady-state ethanol yield (glucose) within a chemostat held at a dilution rate of 0.15/hrs originally derived from a magnesium-limitation of 50uM.

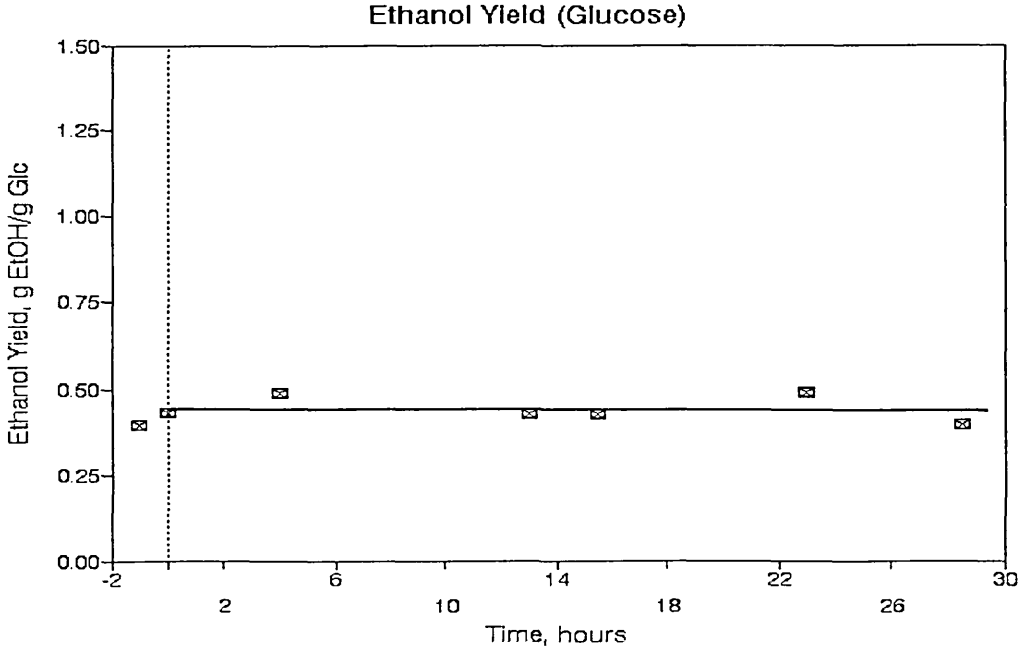


Figure 3.89: The influence of a 140uM magnesium-pulse on the steady-state biomass yield (magnesium) within a chemostat held at a dilution rate of 0.15/hrs originally derived from a magnesium-limitation of 50uM.

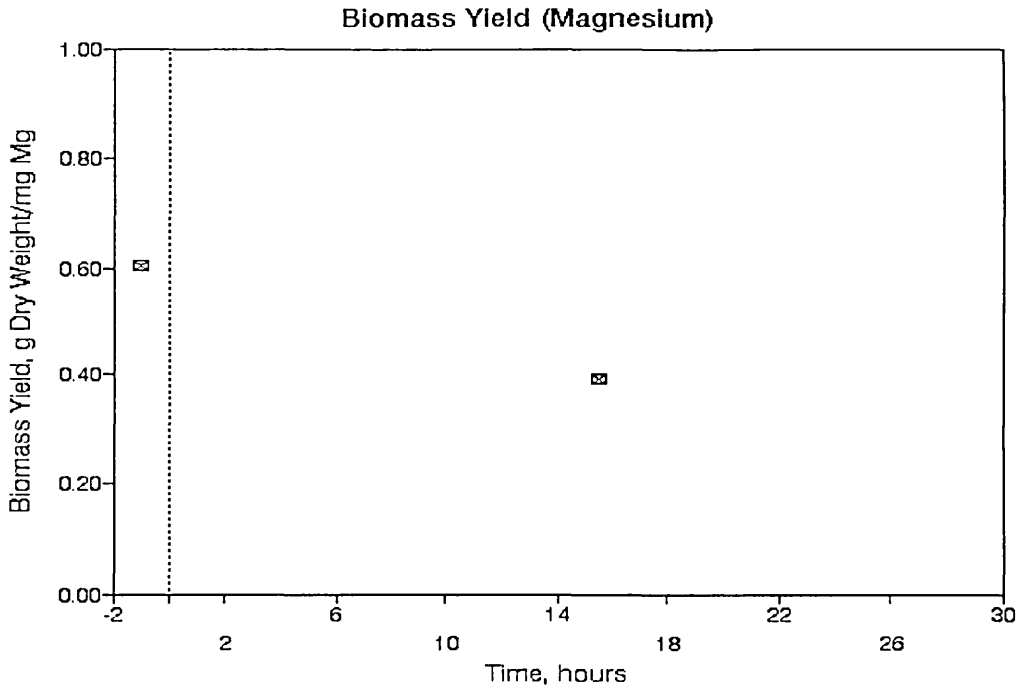


Figure 3.90: The influence of a 140uM magnesium-pulse on the steady-state biomass yield (magnesium) within a chemostat held at a dilution rate of 0.15/hrs originally derived from a magnesium-limitation of 50uM.

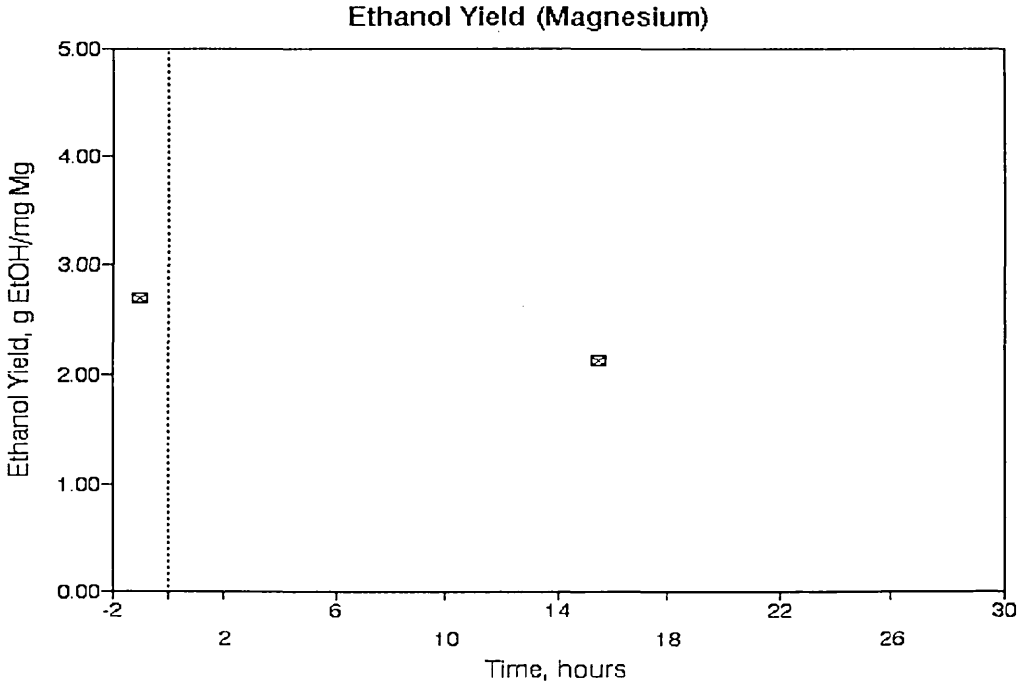


Figure 3.91: The influence of a 140uM magnesium-pulse on the steady-state ethanol production, expressed as a ratio of cell concentration, within a chemostat held at a dilution rate of 0.15/hrs originally derived from a magnesium-limitation of 50uM.

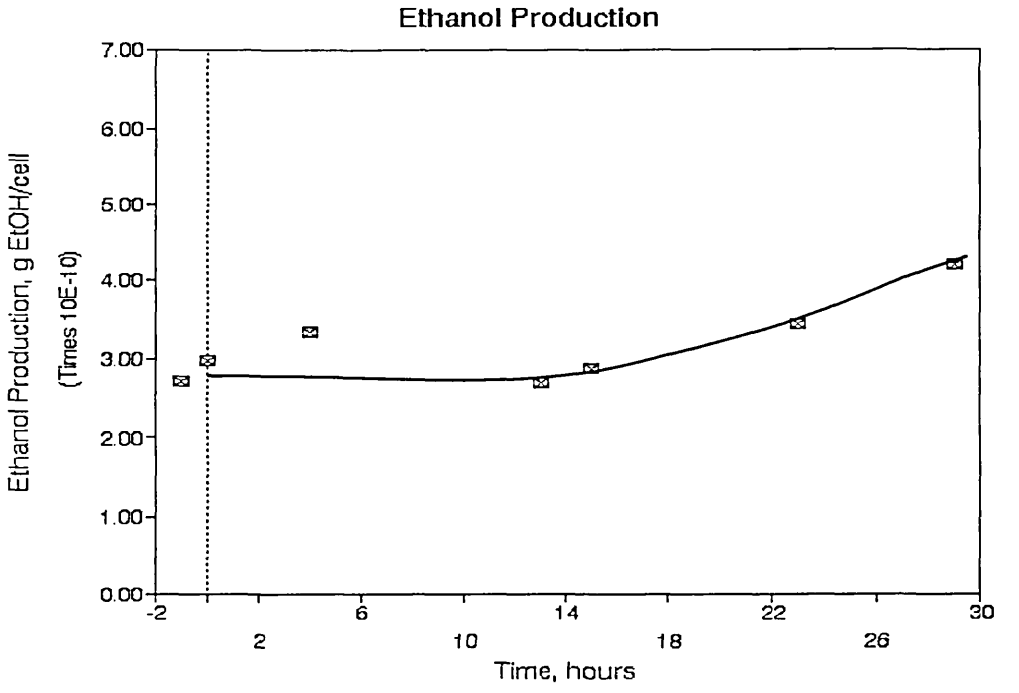


Figure 3.92: The influence of a 140uM magnesium-pulse on the steady-state ethanol production, expressed as ratio of dry weight, within a chemostat held at a dilution rate of 0.15/hrs originally derived from a magnesium-limitation of 50uM.

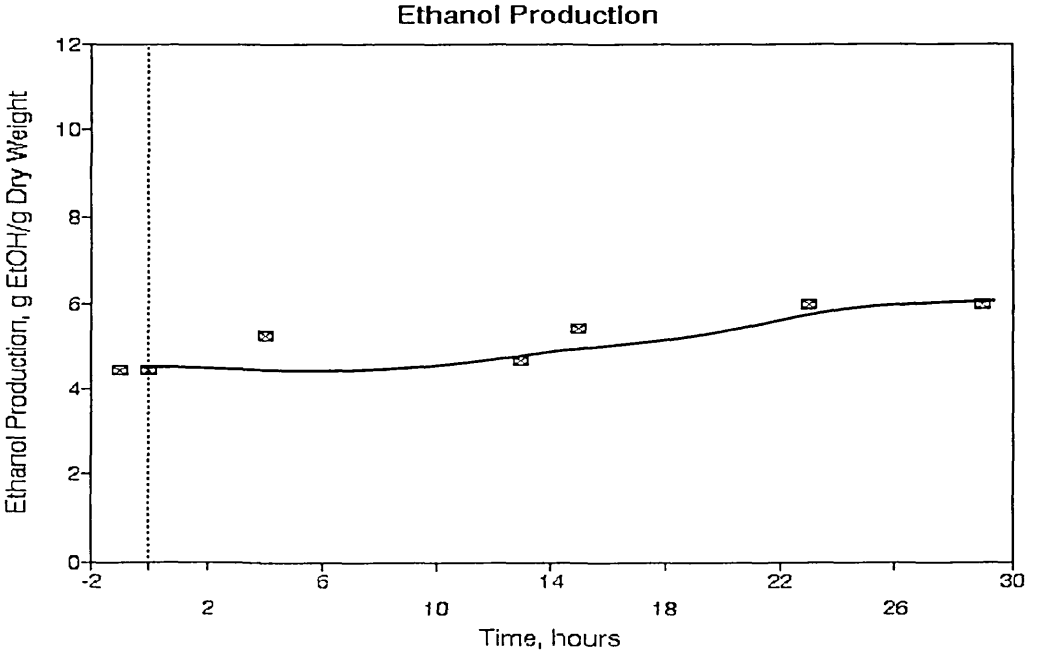


Figure 3.93: The influence of a 140uM magnesium-pulse on the steady-state glucose consumption, expressed as a ratio of cell concentration, within a chemostat held at a dilution rate of 0.15/hrs originally derived from a magnesium-limitation of 50uM.

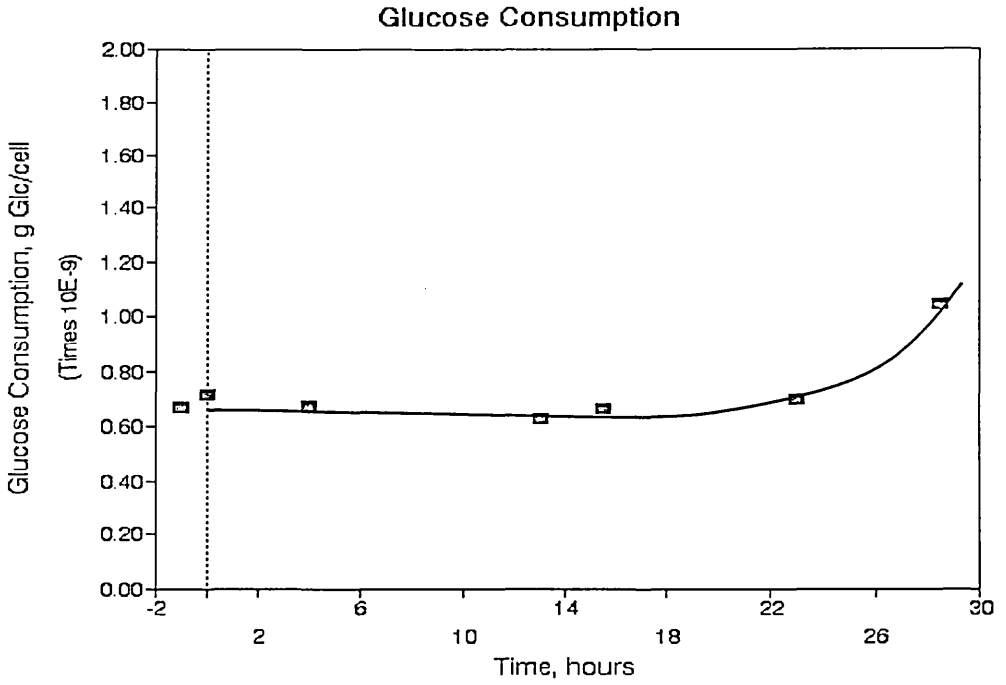
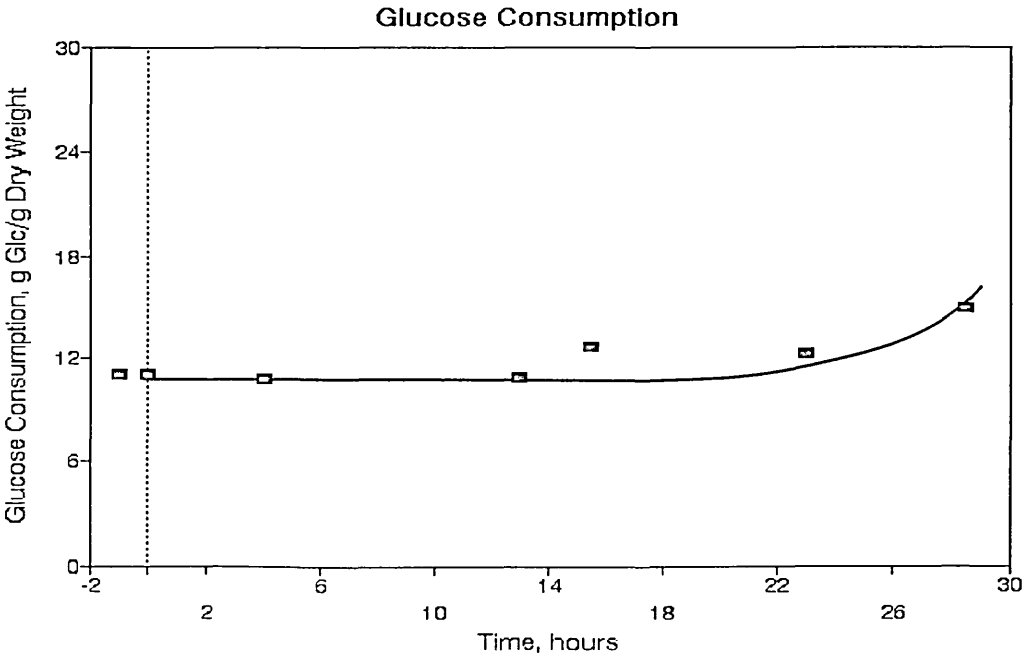


Figure 3.94: The influence of a 140uM magnesium-pulse on the steady-state glucose consumption, expressed as a ratio of dry weight, within a chemostat held at a dilution rate of 0.15/hrs originally derived from a magnesium-limitation of 50uM.



3.2.4. The Effect of a Magnesium Spike on the Chemostat Steady-State

3.2.4.1. Introduction

The introduction of a magnesium pulse to the cell population within the chemostat appeared to permit a period of growth similar to a batch growth curve although there appeared to be no immediate effect on the cells' metabolism. However, if the increase in magnesium within the chemostat occurs rapidly then it is expected that only the faster, more short-term metabolic processes will be able to respond whereas the slower, long-term processes of growth will not be able to utilize the magnesium before it is washed out of the system. Uptake patterns from batch studies would suggest that despite this, the cell population present at the introduction of the spike will have time to utilize the extra magnesium and therefore a distinction between the metabolic response and the growth response of the cell population may not be identifiable.

3.2.4.2. Methods

The chemostat set-up has been detailed in section 2.2.1; the medium preparation in section 2.2.2; the inoculum preparation in section 2.2.3; and the analyses of samples in section 2.3.

Continuous culture was initiated as described in section 2.2.3 with the exception that the dilution rate was set immediately at $0.15hrs^{-1}$ and the chemostat allowed to equilibrate for four fermenter volumes. The chemostat was then sampled, allowed to recover, sampled a second time and again a re-equilibration permitted before a third sample was removed from the chemostat whereupon the system was allowed to establish a steady state over four fermenter volumes.

Once steady-state had been achieved, 2mls of the magnesium stock solution was injected into the chemostat using a sterile syringe. This immediately raised the magnesium concentration within the chemostat from a residual level to 160µM (Figure 3.101) whilst maintaining the feed concentration within the mixing vessel at 50µM (Figure 3.102). No changes were made to any of the pumping rates. This procedure was repeated for a magnesium spike of 240µM.

Again, normal sampling according to section 2.2.3 could not be followed and therefore the methodology detailed in section 3.2.3.2 was used.

3.2.4.3. Results and Discussion

The Effect of a 160μM Magnesium Spike on the Chemostat Steady-State

The results detail several directly measured and derived fermentative and respirative aspects of the continuous, magnesium-limited growth of a cell population of *S. cerevisiae* responding to a spike of magnesium directly into the chemostat.

Changes in the measured parameters (cell concentration, medium absorbance, cell dry weight, mean cell volume, medium ethanol, glucose, and magnesium, cellular magnesium levels, oxygen uptake rate, medium pH and oxygen levels) as a function of the magnesium-controlled growth rate are shown in Figures 3.95, 3.96, 3.97, 3.98, 3.99, 3.100, 3.101, 3.102, 3.103, 3.104, 3.105, 3.106, 3.107, and 3.108 respectively. The effect of growth rate on the derived variables of biomass and ethanol yields (glucose and magnesium), ethanol production (cell and dry weight) and glucose uptake (cell and dry weight) are shown in Figures 3.109, 3.110, 3.111, 3.112, 3.113, 3.114, 3.115, and 3.116 respectively. The introduction of the spike is indicated by the dotted line at zero hours with the steady-state parameter values being given prior to this zero time. The results for the magnesium spike of 240μM are not presented here as the trends are identical to those appearing for the 160μM magnesium spike.

The most immediate result of introducing a spike of magnesium to the chemostat was the rapid increase in cell numbers (Figure 3.95) with the cell concentration reaching a maximum after 7hrs as contrasted with the cell concentration during the pulse experiment (Figure 3.73) which reached a maximum after 16hrs.

In the case for magnesium, it is already known that under limitation, cells of *S. pombe* require a critical intracellular concentration of magnesium to be reached before cell division can be initiated (Walker and Duffus, 1980) and therefore it is possible that

a similar situation is to be found in *S. cerevisiae*; a hypothesis already suggested from the batch results (section 3.1.1.3).

The fresh magnesium introduced into the medium (Figure 3.101) is quickly taken up by the cells (Figures 3.103 and 3.104), permitting rapid cell division that results in an immediate increase in cell numbers. (A corollary to the increase in cell numbers is the significant decline in the pH of the medium (Figure 3.107) which indicates an increase in metabolic activity due to proton extrusion by an active ATPase.) The mean cell volume drops sharply within the first two hours (Figure 3.98) as a result of this cell division. The many smaller daughter cells produced then grow on an individual basis over the next four hours (Figure 3.97 and Figure 3.98) whereupon a second generation of cells is produced, as indicated by the second drop in cell volume from the 6-to-10hour mark (Figure 3.98).

Hence, the release from magnesium-limitation by the spike does not result in an immediate increase in biomass; i.e. synthesis of cellular material; but instead releases limitations within the cell cycle resulting in cell division with the individual mother cells having to effectively half their cell material to achieve cell replication.

The cell dry weight (Figure 3.97) and optical density (Figure 3.96) lag behind the cell concentration in reaching their maxima (12-14hrs), as is to be expected from these deductions, but bear a close similarity to the results of the magnesium pulse (16hrs). However, the effect of the magnesium-spike does not result in a distinct washout of cells (Figure 3.95) in the manner exhibited by the magnesium-pulse (Figure 3.73) but rather the cell numbers take nearly twice as long in the spike experiment to fall back to their original level. This would indicate that the washout effect of the chemostat is being more strongly compensated for by the spike's cell population and that the spike's effect on cell replication is more pronounced than that of the pulse.

That only a relatively small quantity of glucose is taken from the medium within the first 6hrs (Figure 3.100), lagging behind the appearance of the new cells, would

suggest that the initial population present does not have time to remove glucose from the medium for cell replication and that it is the daughter cells that consume the glucose for the production of cell constituents and hence the second generation of cells.

Differences between the glucose consumption based on dry weight (Figure 3.116) and the consumption on a per cell basis (Figure 3.115) are noticeable. The former reveals a slight increase in uptake over the first 6hrs whereas the latter is constant with only a slight decrease occurring. Hence, the glucose levels within the chemostat decrease whilst the cell concentration increases and the dry weight remains constant; this results in the glucose consumption per cell remaining constant whilst glucose consumption per gram increases.

The quantity of new ethanol appearing in the medium (Figure 3.99) is lower than might have been expected if the cells were responding to the magnesium spike on a solely metabolic level but would again appear to support the hypothesis that the primary result of the magnesium was a release of the cell cycle from limitation.

The results for the ethanol production of the cell population (Figures 3.113 and 3.114) again reveal a difference based on dry weight and cell number calculations. On a per cell basis, the ethanol production value falls very quickly to a minimum 6hrs after the spike whereas on a dry weight basis the ethanol production remains fairly constant for the 6hrs. Hence, the ethanol levels within the chemostat remain constant whilst the cell concentration increases and the dry weight remains constant; this results in the ethanol production per cell decreasing whilst ethanol production per gram remains constant.

If the reduction in medium oxygen levels (Figure 3.118) is to be accounted for in a similar manner then it would be expected that as the oxygen levels within the chemostat decrease whilst the cell concentration increases and the dry weight remains constant then the oxygen uptake rate per cell would remain constant whilst the oxygen uptake rate per gram would increase. From Figures 3.105 and 3.106, it can be seen that

this is not the case and that the uptake rate per cell decreases sharply and the rate per gram of dry weight remains constant.

This difference is accounted for by the rapidity with which the cell concentration increases and the comparative slowness with which the dry weight increases. Thus, the spike of magnesium does not alter the oxygen uptake rate of the total cell dry weight present within the culture but in permitting cell division to occur, the uptake rate on a per cell basis is effectively lowered; i.e. the biomass within the mother cells responsible for the oxygen uptake is divided into the new cells without any compensation occurring. Hence, the newly synthesized daughter cells have a lower requirement for oxygen that steadily increases as individuals increase the quantity of cell constituents.

Curves for the biomass and ethanol yield from glucose are shown in Figures 3.109 and 3.110 with both variables declining rapidly within the first two hours after the spike only to recover to their original values within 9 hours.

Data for the biomass and ethanol yield from magnesium (Figures 3.111 and 3.112) were difficult to calculate given that the system is dynamic in nature. Individual data points would have to be calculated by taking into account that the magnesium from the spike will not only be utilized by the cells but will also be washed out of the chemostat. Concurrently, the washout of the spike magnesium would be ameliorated by the influx of fresh magnesium from the reservoir medium. In hindsight, it may have been more informative to have ceased the influx of fresh medium to the chemostat for the duration of the spike as this would have reduced the number of complicating variables by effectively 'fixing' the amount of available magnesium, glucose and other nutrients and at the same time ensuring that all the products of the spike, principally ethanol and biomass, attained their maximum concentrations.

However, given that this were not the case, it can be argued that in allowing the chemostat to remain operational during the spike all the parameters were subjected to the same conditions and therefore although the levels of various products may not have

attained their true concentrations the trends are nonetheless valid indicators of the cell population's response to the spike.

Hence, the biomass and ethanol yield co-efficients were calculated for the data point at which the dry weight and ethanol had reached their maximum values with the assumption that all the magnesium from the spike had been made available to the cell population as the data points used corresponded to the time when all the magnesium from the spike had disappeared from the medium. The data for the pulse experiment was treated in a similar manner.

From these assumptions, it can be seen from Figures 3.111 and 3.112 that after 7hrs. both the biomass and the ethanol yield have fallen significantly as a result of the addition of magnesium although it is felt inadvisable to deduce to much from such tentative calculations.

Figure 3.95: The influence of a 160uM magnesium-spike on the steady-state cell concentration within a chemostat held at a dilution rate of 0.15/hrs originally derived from a magnesium-limitation of 50uM.

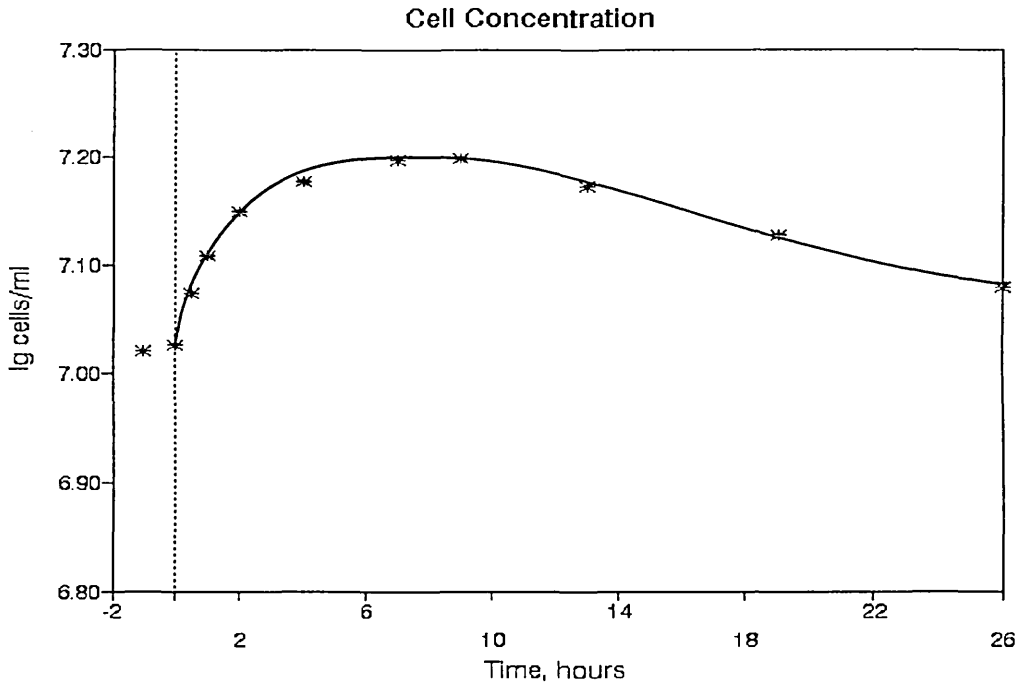


Figure 3.96: The influence of a 160uM magnesium-spike on the steady-state optical density within a chemostat held at a dilution rate of 0.15/hrs originally derived from a magnesium-limitation of 50uM.

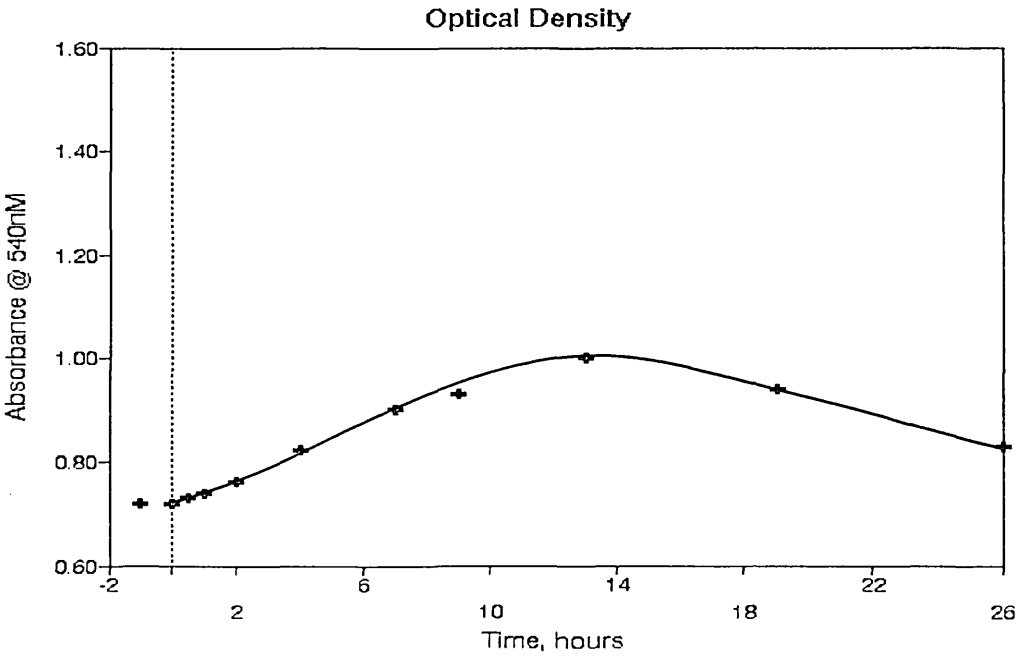


Figure 3.97: The influence of a 160uM magnesium-spike on the steady-state dry cell weight within a chemostat held at a dilution rate of 0.15/hrs originally derived from a magnesium-limitation of 50uM.

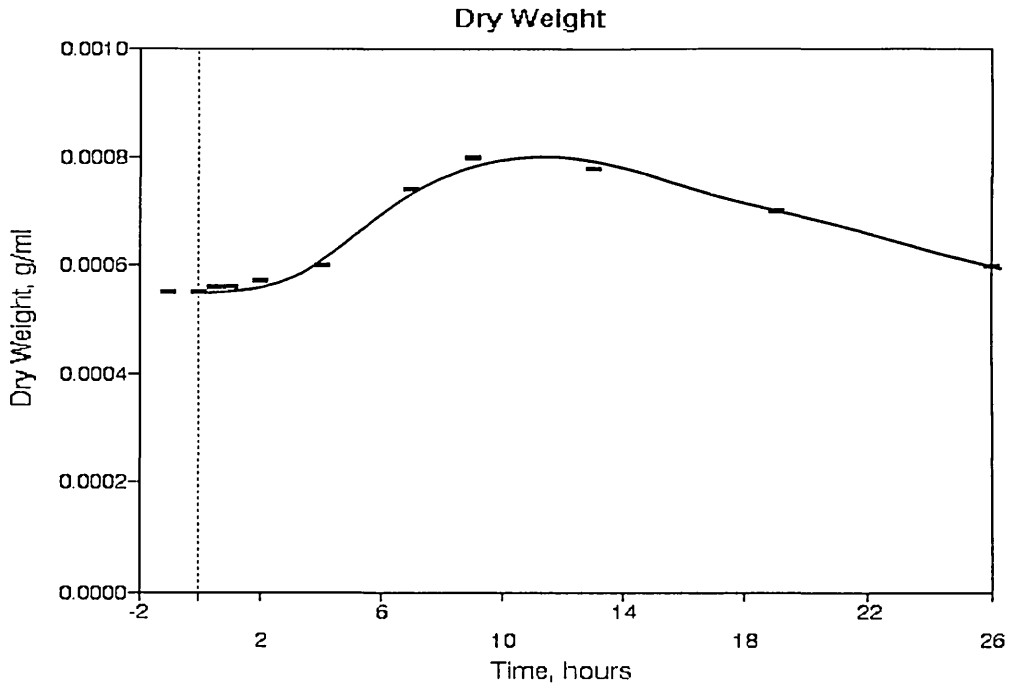


Figure 3.98: The influence of a 160uM magnesium-spike on the steady-state mean cell volume within a chemostat held at a dilution rate of 0.15/hrs originally derived from a magnesium-limitation of 50uM.

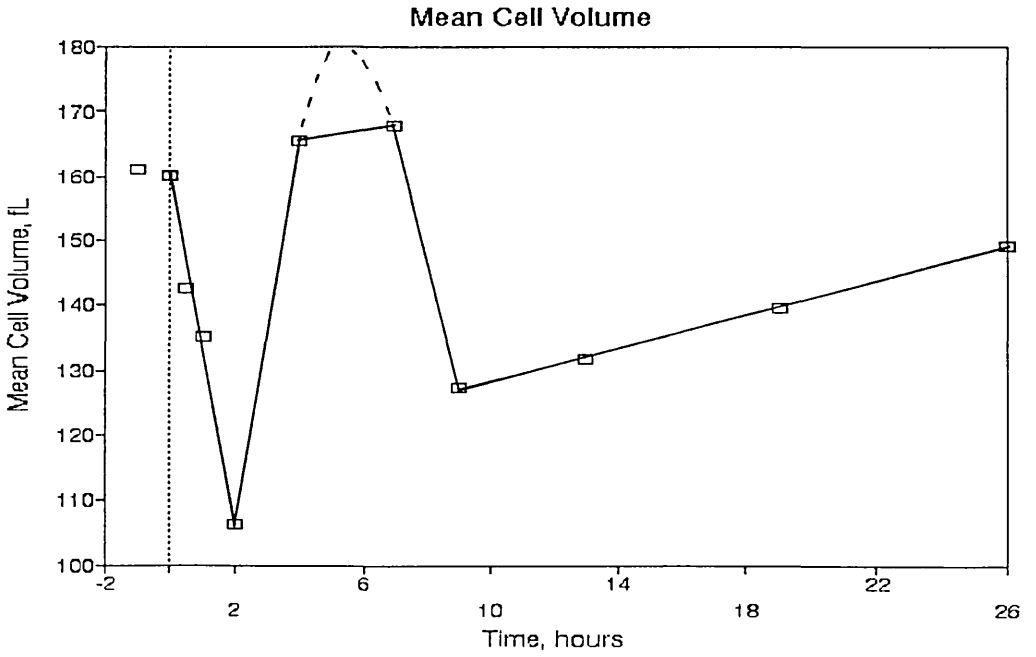


Figure 3.99: The influence of a 160uM magnesium-spike on the steady-state ethanol concentration within a chemostat held at a dilution rate of 0.15/hrs originally derived from a magnesium-limitation of 50uM.

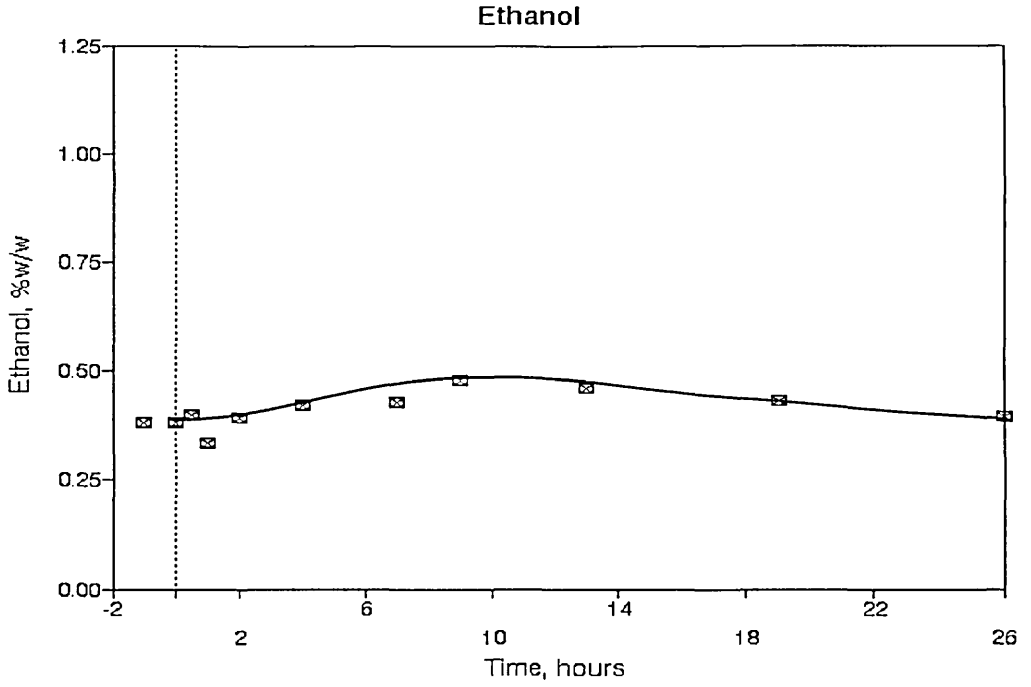


Figure 3.100: The influence of a 160uM magnesium-spike on the steady-state glucose concentration within a chemostat held at a dilution rate of 0.15/hrs originally derived from a magnesium-limitation of 50uM.

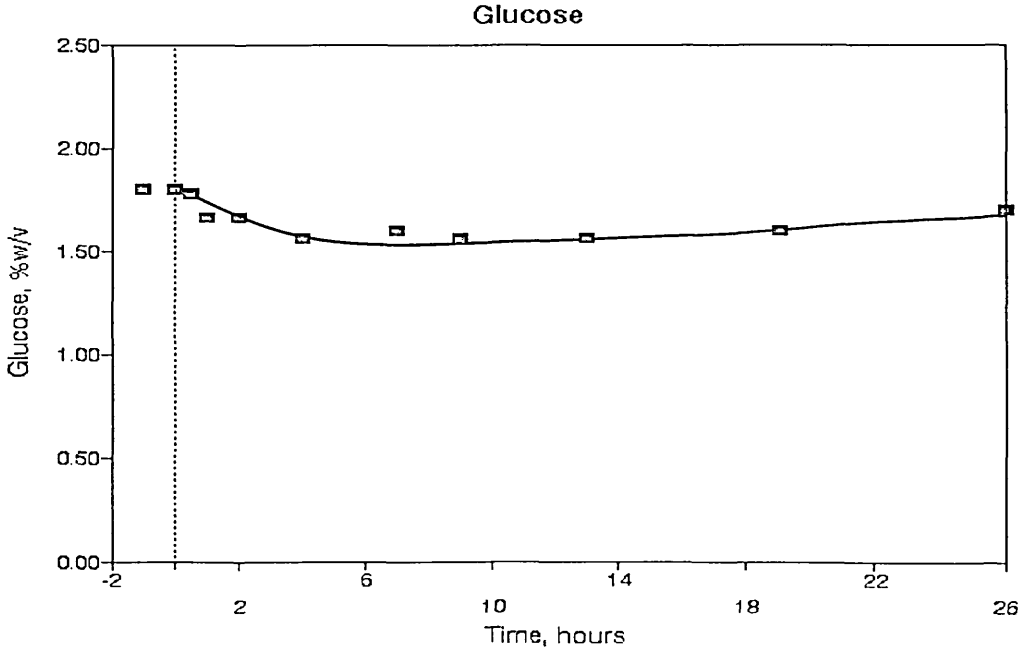


Figure 3.101: The influence of a 160uM magnesium-spike on the steady-state magnesium concentration within a chemostat held at a dilution rate of 0.15/hrs originally derived from a magnesium-limitation of 50uM.

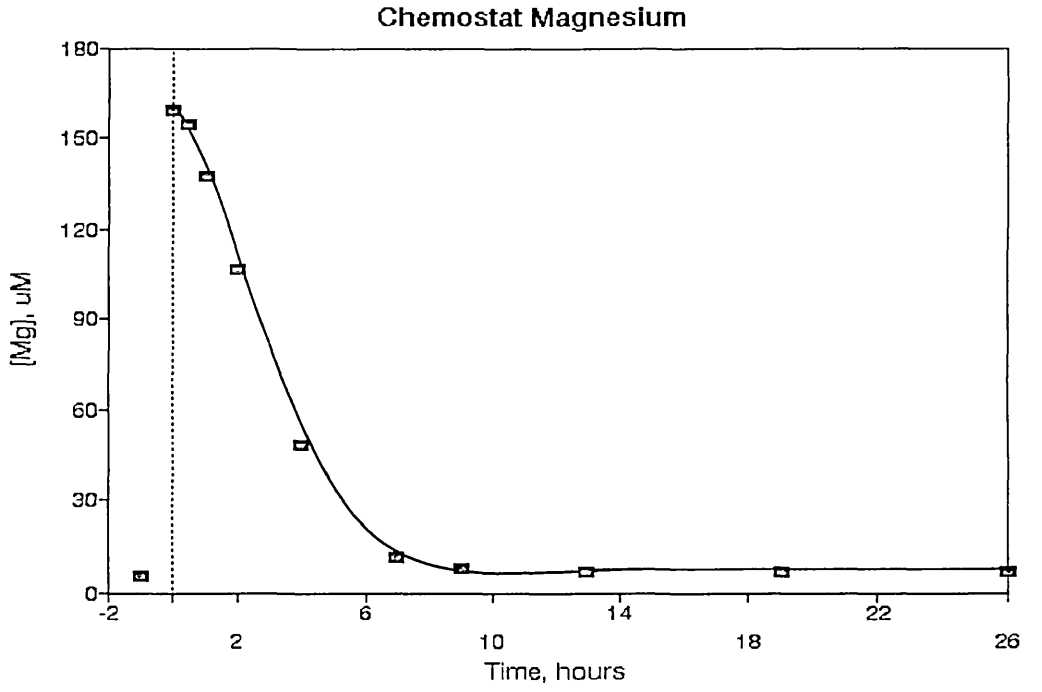


Figure 3.102: The magnesium concentration within the reservoir medium during the magnesium-spike within the chemostat.

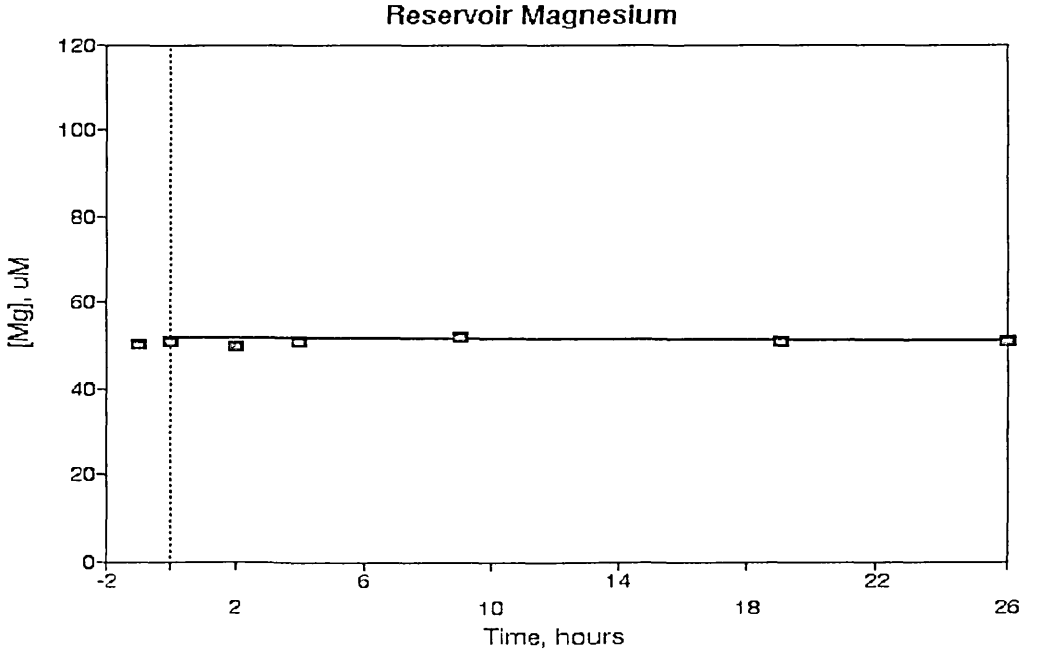


Figure 3.103: The influence of a 160uM magnesium-spike on the steady-state cell magnesium concentration, expressed as a ratio of cell concentration, within a chemostat held at a dilution rate of 0.15/hrs originally derived from a magnesium-limitation of 50uM.

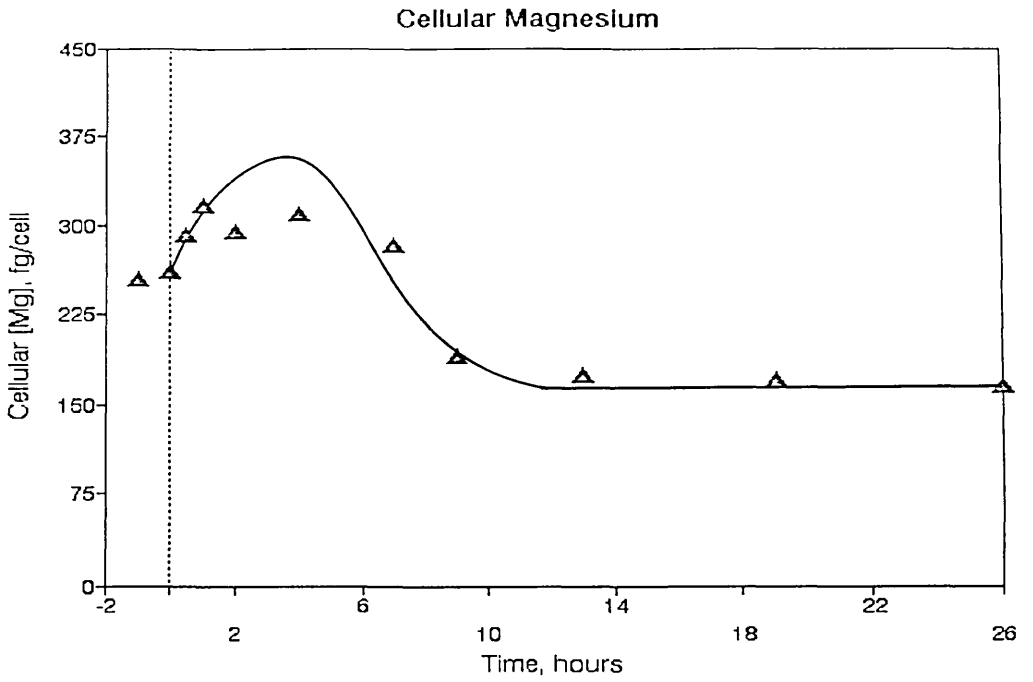


Figure 3.104: The influence of a 160uM magnesium-spike on the steady-state cell magnesium concentration, expressed as a ratio of dry weight, within a chemostat held at a dilution rate of 0.15/hrs originally derived from a magnesium-limitation of 50uM.

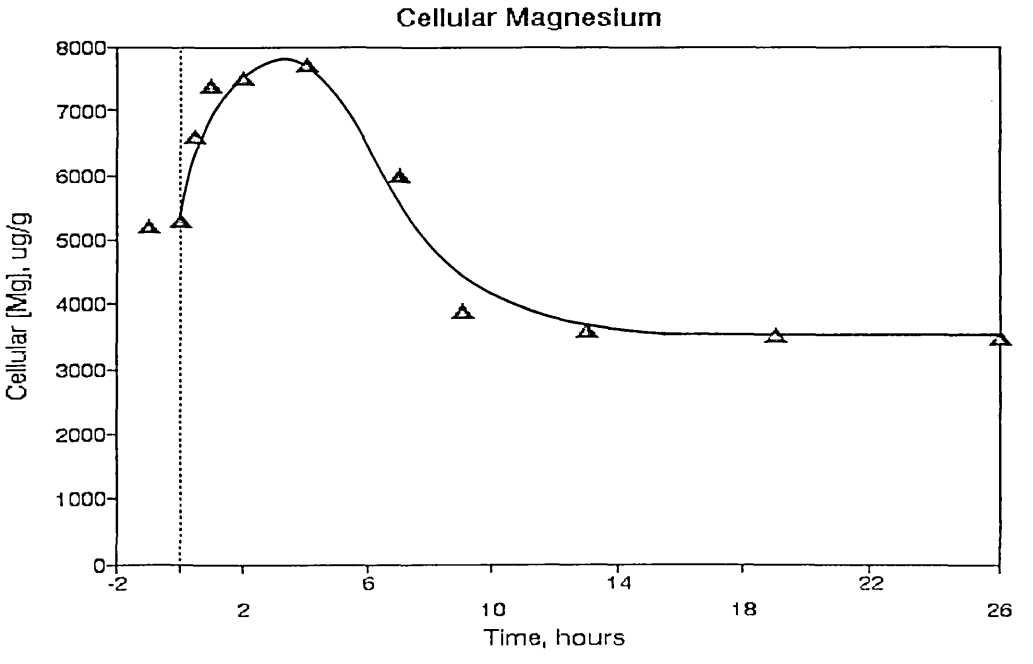


Figure 3.105: The influence of a 160uM magnesium-spike on the steady-state oxygen uptake rate, expressed as a ratio of cell concentration, within a chemostat held at a dilution rate of 0.15/hrs originally derived from a magnesium-limitation of 50uM.

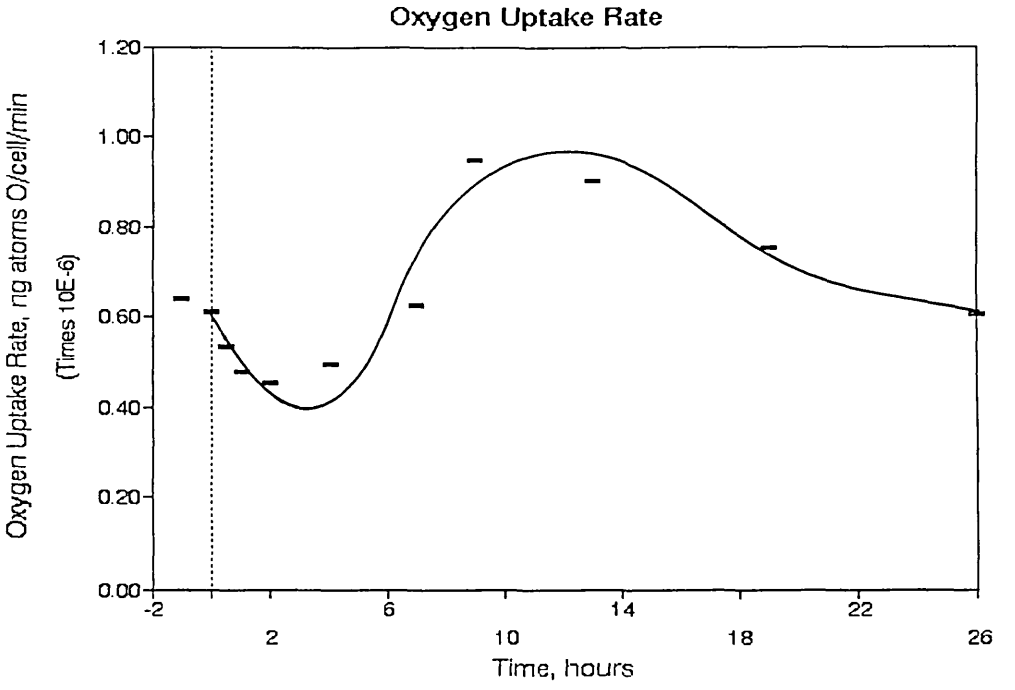


Figure 3.106: The influence of a 160uM magnesium-spike on the steady-state oxygen uptake rate, expressed as a ratio of dry weight, within a chemostat held at a dilution rate of 0.15/hrs originally derived from a magnesium-limitation of 50uM.

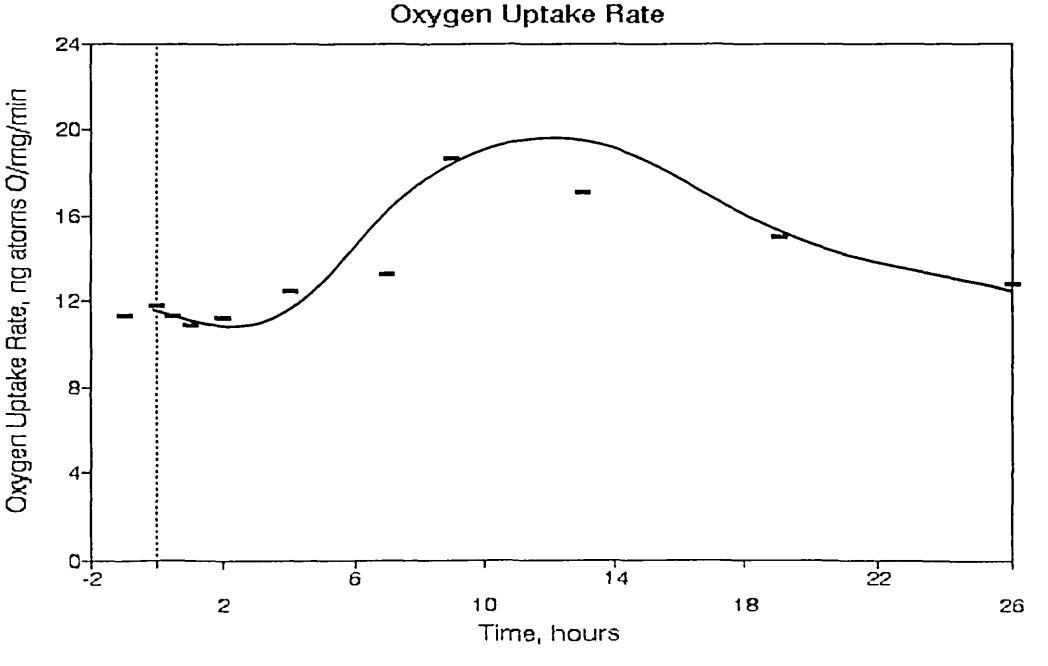


Figure 3.107: The influence of a 160uM magnesium-spike on the steady-state pH within a chemostat held at a dilution rate of 0.15/hrs originally derived from a magnesium-limitation of 50uM.

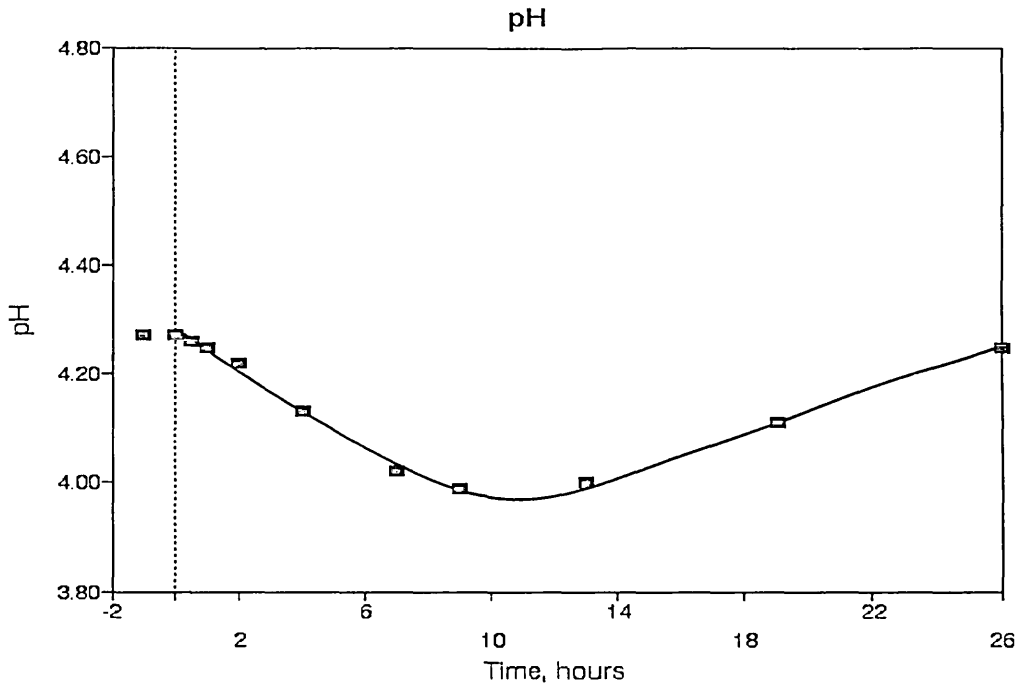


Figure 3.108: The influence of a 160uM magnesium-spike on the steady-state oxygen levels within a chemostat held at a dilution rate of 0.15/hrs originally derived from a magnesium-limitation of 50uM.

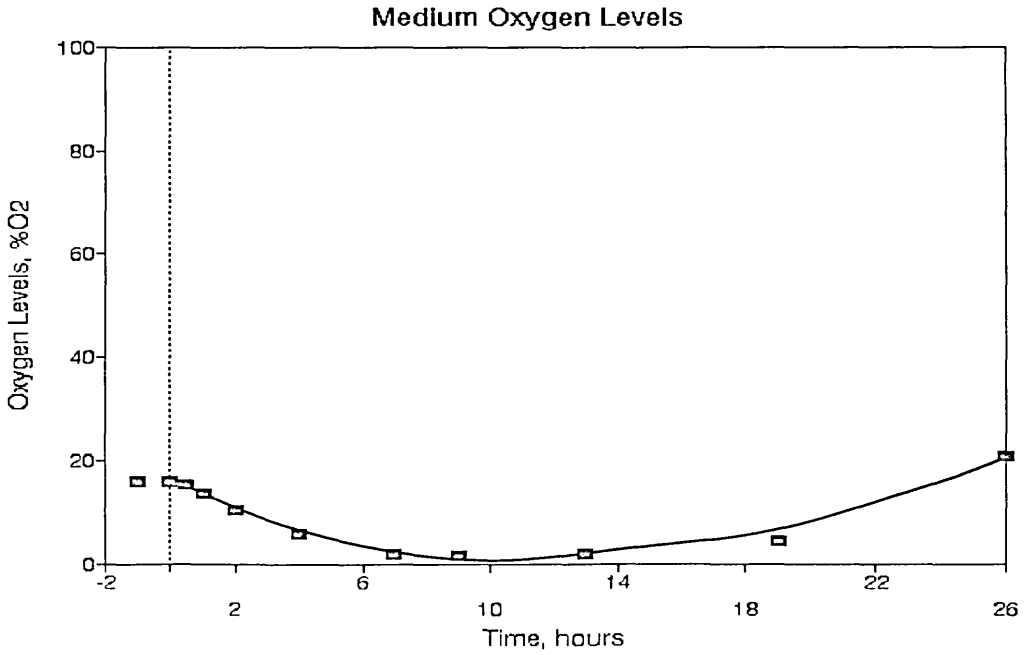


Figure 3.109: The influence of a 160uM magnesium-spike on the steady-state biomass yield (glucose) within a chemostat held at a dilution rate of 0.15/hrs originally derived from a magnesium-limitation of 50uM.

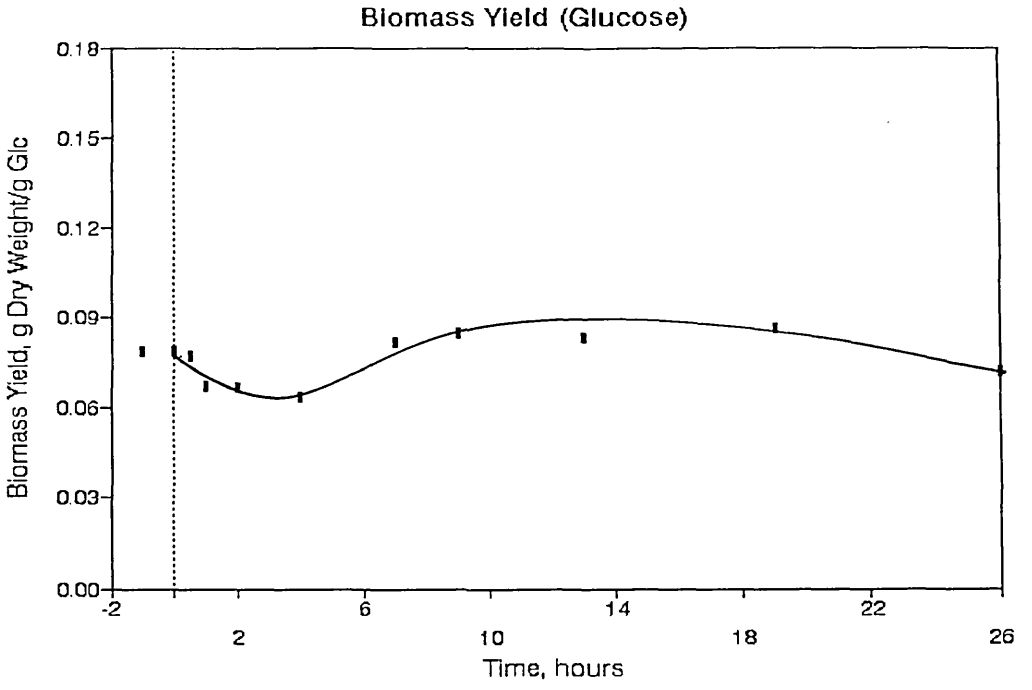


Figure 3.110: The influence of a 160uM magnesium-spike on the steady-state ethanol yield (glucose) within a chemostat held at a dilution rate of 0.15/hrs originally derived from a magnesium-limitation of 50uM.

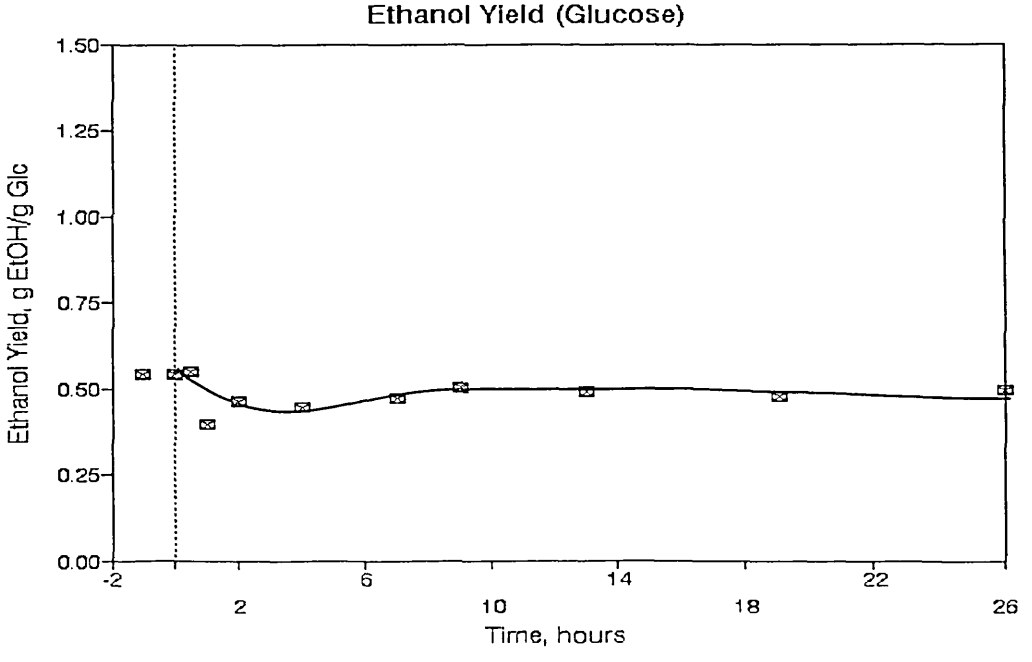


Figure 3.111: The influence of a 160uM magnesium-spike on the steady-state biomass yield (magnesium) within a chemostat held at a dilution rate of 0.15/hrs originally derived from a magnesium-limitation of 50uM.

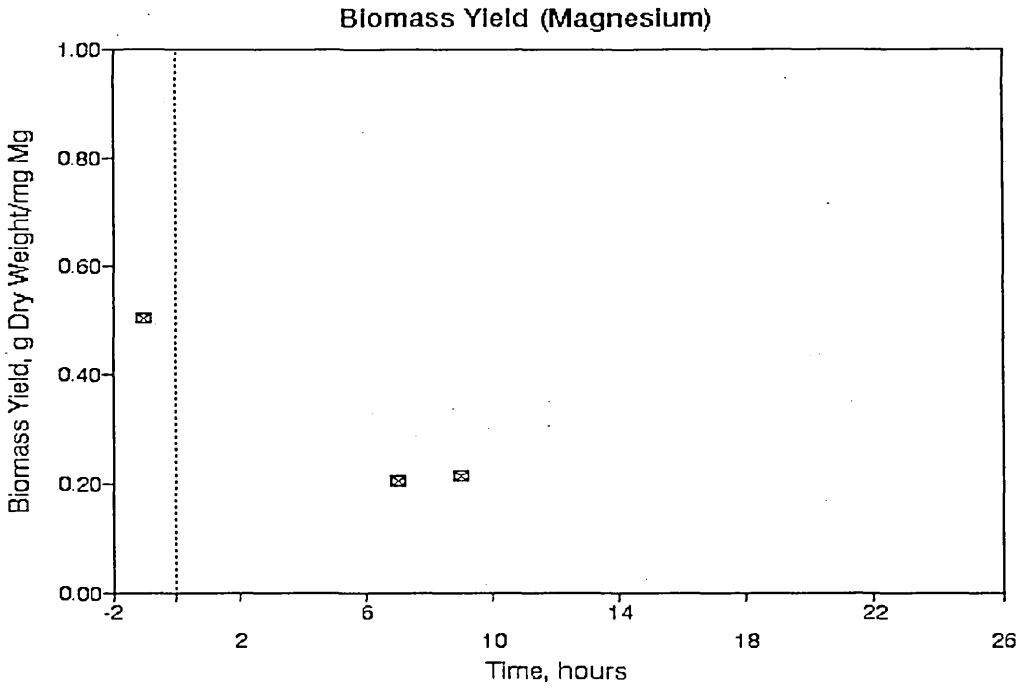


Figure 3.112: The influence of a 160uM magnesium-spike on the steady-state ethanol yield (magnesium) within a chemostat held at a dilution rate of 0.15/hrs originally derived from a magnesium-limitation of 50uM.

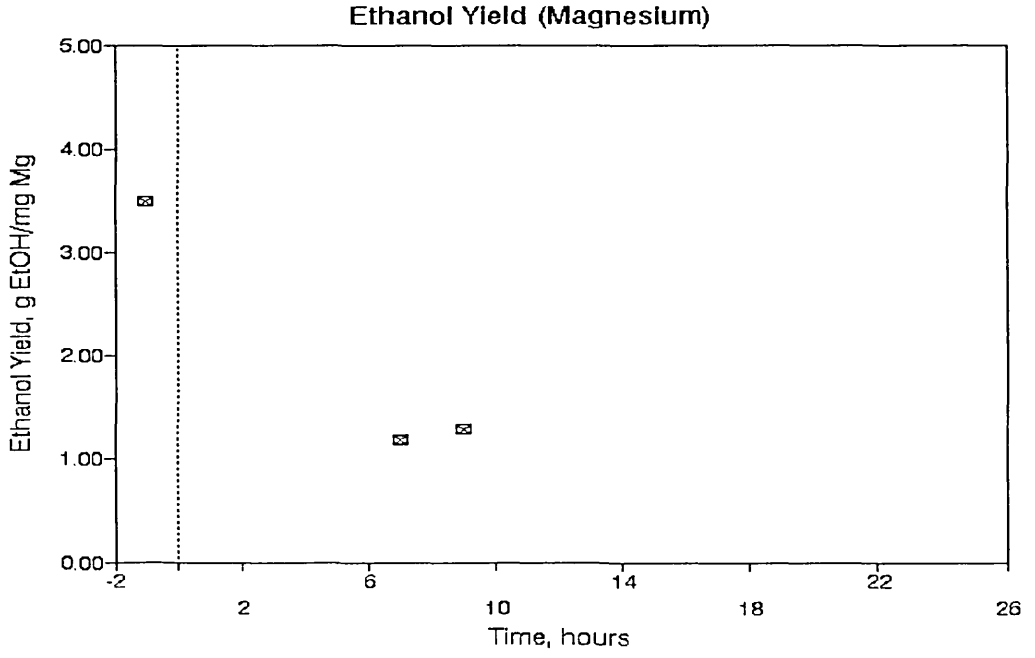


Figure 3.113: The influence of a 160uM magnesium-spike on the steady-state ethanol production, expressed as a ratio of cell concentration, within a chemostat held at a dilution rate of 0.15/hrs originally derived from a magnesium-limitation of 50uM.

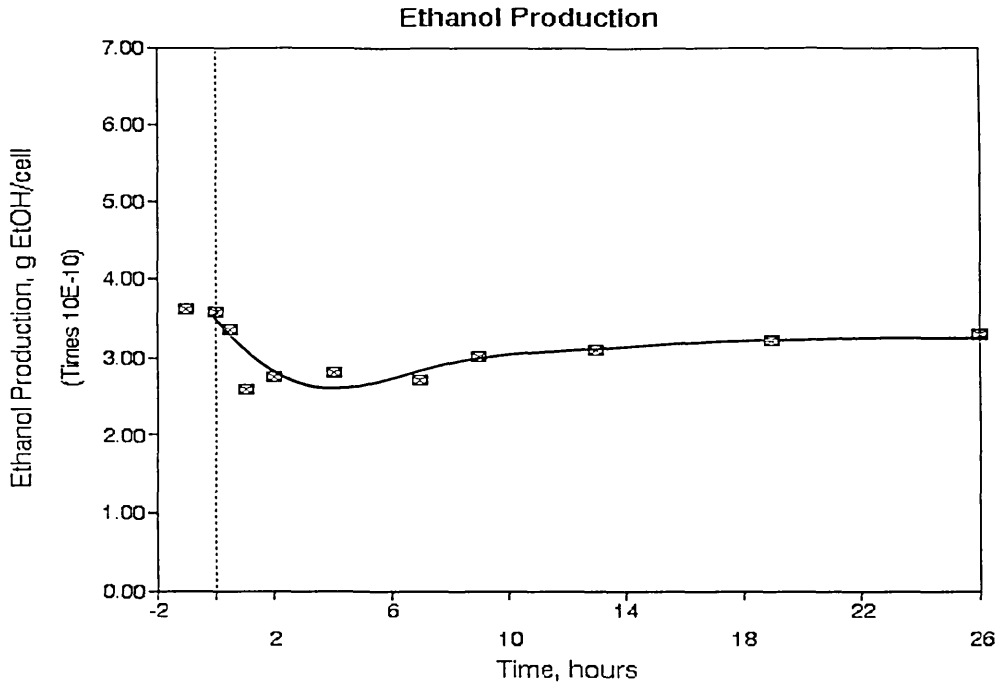


Figure 3.114: The influence of a 160uM magnesium-spike on the steady-state ethanol production, expressed as a ratio of dry weight, within a chemostat held at a dilution rate of 0.15/hrs originally derived from a magnesium-limitation of 50uM.

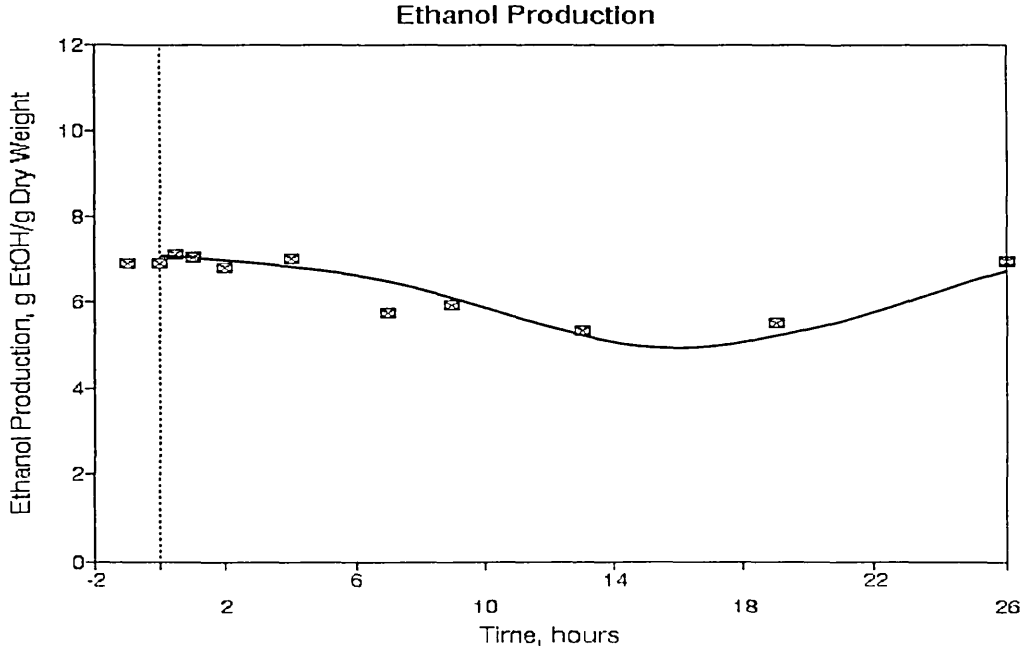


Figure 3.115: The influence of a 160uM magnesium-spike on the steady-state glucose consumption, expressed as a ratio of cell concentration, within a chemostat held at a dilution rate of 0.15/hrs originally derived from a magnesium-limitation of 50uM.

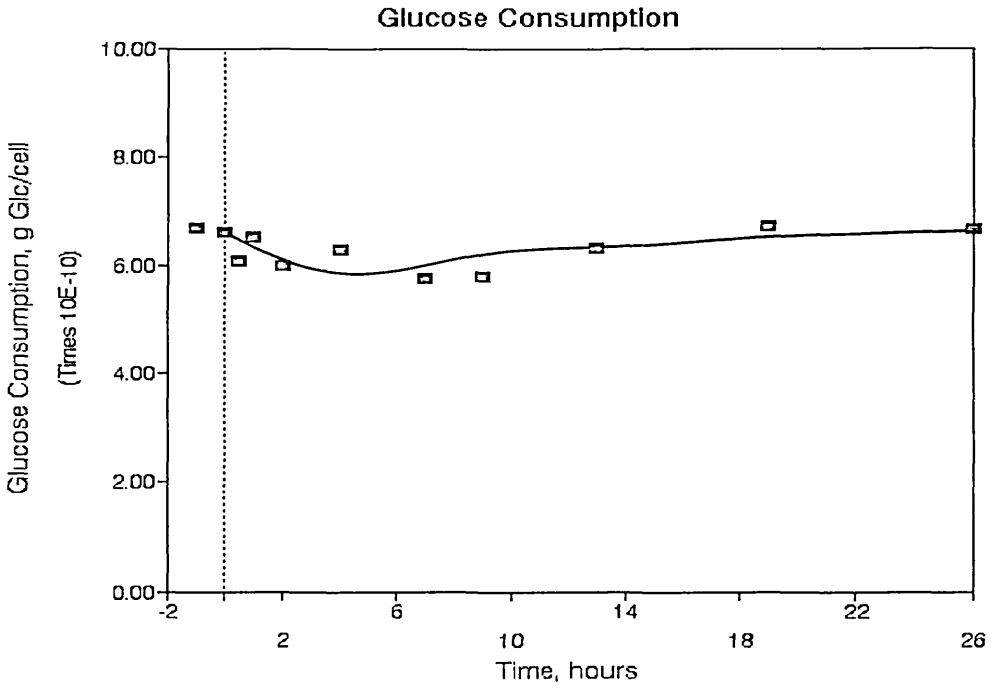
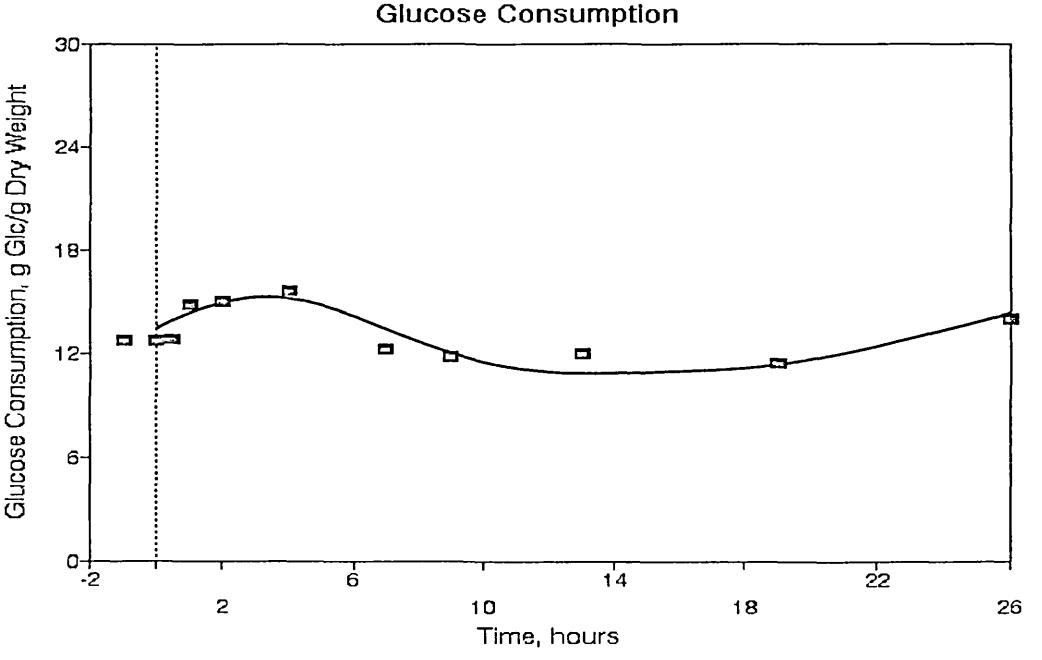


Figure 3.116: The influence of a 160uM magnesium-spike on the steady-state glucose consumption, expressed as a ratio of dry weight, within a chemostat held at a dilution rate of 0.15/hrs originally derived from a magnesium-limitation of 50uM.



CHAPTER 4: DISCUSSION

The growth response of a batch culture of *Saccharomyces cerevisiae* to magnesium-limitation under conditions of glucose-repression was shown to be hyperbolic, thus indicating a Monod relationship. The maximum growth rate was found to be $0.20hrs^{-1}$ whilst K_S was equal to 36μM and the yield co-efficient at $^1/_2\mu_{max}$ approximately equal to 1.9grams of cells formed per milligram of magnesium removed from the medium. The study of the steady-state for any given limiting nutrient is started by delineating these growth parameters (Dawson, 1985); the first two parameters indicate how fast an organism grows whilst the latter two reveal how effectively the substrate is being used (Fiechter *et al.*, 1987).

Magnesium uptake patterns were obtained and indicated a pattern of uptake and release that corresponded to the increase and decrease in metabolic activities of the population further indicating the highly regulated nature of the ion within the yeast cell (Jones and Greenfield, 1984). The consistent finding of release patterns questions the concept of 'nutrient-limitation' and the effect of the patterns on the gradual loss of fermentative activity. Dombek and Ingram (1986) found that the magnesium supplementation of YEPD prolonged exponential growth; patterns of uptake were similar but did not reveal release patterns as are reported here. However, the fermentations carried out by Dombek and Ingram (1986) were considerably slower than these batch studies with the exhaustion of glucose and completion of ethanol production not occurring until over 55hours of elapsed fermentation time. From the results presented here this is when the onset of magnesium release would be expected but the experiments of those authors ended at 70hours. Differences in medium types may also greatly influence magnesium uptake and release patterns.

The suggested downward revision of the yeasts' exogenous magnesium requirements from 2-4mM (Jones and Greenfield, 1984) to 0.5mM (Jones, 1986) is

confirmed, with a specific level of 0.65mM for a minimal medium at 2.5%w/v glucose being recommended.

Hence, the characterization of the magnesium requirements of *S. cerevisiae* during growth and fermentation within a batch culture has been achieved as has the establishment of the growth-limiting magnesium concentration.

The matching of the exogenous magnesium concentration within the main fermenting medium to the inoculum magnesium concentration is shown to be of importance in influencing the fermentation performance of the cell population although an excess of magnesium within the inoculating medium can neutralise the detrimental effects of a low magnesium concentration within the main fermenting medium on the fermentation parameters of the cell population.

The cellular ethanol productivity may be more greatly influenced by the inoculum magnesium concentration than by the concentration within the main fermenting medium itself although much further work is required to prove this conclusively. The cells release of the magnesium back into the medium implies that the timing of the transfer of the inoculum may be critical in influencing the cellular ethanol productivity and also partially explains the anecdotal evidence that the use of an exponential-phase inoculating culture minimizes the length of the lag-phase within the main culture.

Due to the economic possibilities of bioethanol as an octane-enhancer for petroleum fuels and as a replacement for these fuels there is obviously a large degree of interest into the basis for the physiological conditions which influence the rate and extent of ethanol production (Casey and Ingledew, 1986; van Uden, 1985; Ingram and Buttke, 1984; Moulin *et al.*, 1984; Leao and van Uden, 1982) and therefore these conditioning experiments suggest a possibly beneficial area of research. The economics of bulk ethanol production are such that any slight improvement of yield by way of environmental optimization is of great interest (Meyer *et al.*, 1985). Results from the simple addition of magnesium to a complex medium were rather neutral but this was

felt to be due to the nature of the molasses. Conditioning experiments were not carried out within a complex medium and thus remain to be explored.

The investigation of the physiological states of *S. cerevisiae* over a range of growth rates using a magnesium-limited chemostat has been carried out with the results in the main agreeing with the trends established from batch culture.

Principally, at growth rates lower than μ_{max}, the cells reveal a physiological state characteristic of non-carbon-limited growth involving an increase in biomass yield from magnesium and a trend from high levels of fermentation at low growth rates to reduced fermentation and increased respiratory activity as the growth rate increases towards μ_{max}. For the formation of any product, growth is usually at the expense of product-formation (Meyer *et al.*, 1985) and therefore these findings have implications for industrial-scale continuous yeast cultures whereby slow growth rates under magnesium limitation would be used for the production of ethanol whilst high growth rates would be used for the production of biomass.

At growth rates greater than μ_{max}, the cells reveal a physiological state characteristic of mycelial growth that is consistent with the involvement of magnesium in cell morphology. That μ_{max} derived from batch cultures was surpassed during the chemostat cultures has already been attributed to inadequate mixing and washout caused by a pseudo-hyphal growth condition of the cells. Although some small degree of 'scum-line' formation was observed it never appeared to become particularly acute. As the maximal growth rate during the batch fermentations under high glucose levels (2.5%) was surpassed at low glucose levels (1%) then it is possible that the chemostat culture surpassed the batch μ_{max} because of the glucose levels. However, at a dilution rate of 0.20hrs^{-1} the residual glucose within the chemostat is 1.75% and therefore this hypothesis is not considered likely.

Magnesium-limited chemostat cultures of *S. pombe* have shown 'physiologically induced' branching of cells (McDonald, unpublished data; cited by McDonald and Tsai, 1989), whilst cell size within prokaryotes is affected by magnesium-limitation (Utkilen,

1984) as is cell size within eukaryotes (Walker & Duffus, 1980). The limitation of phosphate to cells of *S. cerevisiae* results in large decreases in $Y_{X/S}$ (Jones & Greenfield, 1984) coupled with altered cell-wall and membrane structure and function through altered lipid metabolism. As magnesium is involved in fatty acid synthesis and phosphate uptake, it is possible that the morphological changes observed in this magnesium-limited chemostat are a secondary metabolic effect of magnesium-deficiency although the reduced level of shielding available to anionic sites, especially concerning membrane integrity and function, is considered a more likely explanation.

The actual values for the biomass yield from glucose are of the same order as have been reported for glucose-limited growth (Fiechter *et al.*, 1987) where low dilution rates yielded 0.50g dry weight (g glc)$^{-1}$ and high dilution rates 0.16g dry weight (g glc)$^{-1}$. However, these results indicate a complete reversal of this trend although apparently the yield did not increase until after μ_{max} had been passed. Upto μ_{max}, the yield obtained approximates to 0.08g dry weight (g glc)$^{-1}$ compared to 0.05g (g)$^{-1}$ within a vitamin-only medium (Hauklie and Lie, 1971; cited by Jones *et al.*, 1981).

The results obtained here do not reflect the findings of glucose-limited chemostat cultures of *S. cerevisiae* where ethanol formation is not observed below a growth rate of 0.30hrs^{-1} despite fermentative capacity still being present. The activity of the fermentative capacity rises sharply with dilution rate concurrent to the appearence of glucose and ethanol within the chemostat (Petrik *et al.*, 1983; Rieger *et al.*, 1983). It has been concluded (Kappeli, 1986) that at high dilution rates in glucose-limited, aerobic cultures, growth is respiro-fermentative whereas low dilution rates give only respiratory activity (Kappeli *et al.*, 1985). The studies conducted here reveal the activity of the fermentative capacity at all growth rates with the activity decreasing as the growth rate is increased.

Hence, major differences exist between the behaviour of yeast populations within magnesium-limited chemostats and glucose-limited chemostats. Within a glucose-limitated chemostat, the lack of catabolite repression at the low growth rates permits the cell population to express it's respiratory capacity and thus the metabolism of the population ranges from pure respiration through to respiro-fermentative behaviour. However, these studies used high glucose levels in an effort to mimic industrial fermentations and therefore only the respiro-fermentative option was open to the cells. Thus it is felt that the results from this study represent changes in the relative contributions of each pathway to the overall state of respiro-fermentative metabolism. Hence, the inferred decrease in respiration without any compensatory rise in fermentation (section 3.2.3.3).

A major finding of this study was that a medium reservoir concentration of 90μM magnesium is too high to give magnesium-limited growth within a chemostat despite expectations to the contrary from the batch studies. Karrer (1978; cited by Fiechter *et al.*, 1981) had found that the critical dilution rate at which glucose repression sets in is strongly dependent on the glucose concentration of the incoming media in the continuous culture. The data presented here suggests that the inlet concentration of magnesium strongly affects the physiological status of the culture possibly resulting in a form of 'double-limitation' to be present at dilution rates less than μ_{max} as the glucose level within the chemostat reached zero and the magnesium level also approached zero.

Cell population characteristics are affected by the nature and concentration of the growth-limiting substrate but the effects should not be considered in isolation as the natures and supply of other, (supposedly) non-limiting nutrients will influence the cell's behaviour, especially the carbon source that is believed to be critically influential (Herbert, 1958). For example, the deviation from the theoretical curve mentioned in section 3.2.2.3: magnesium limitation at 50μM, was not observed for the ammonium-limitation of *Torulopsis utilis* if glycerol was substituted for glucose as the carbon and energy source (Herbert, 1958).

Studies on the metabolic behaviour of *S. cerevisiae* when released from magnesium-limitation within a chemostat have been carried out and indicate fixed, describable responses.

The response of the chemostat population to a pulse of magnesium is an inverted hyperbolic increase in cell concentration that superficially resembles a batch-phase growth curve whereas the response of the chemostat population appears to be more directly related to cell division with individual growth occurring later as a sequential or secondary response.

Thus, the manner in which magnesium-limitation is lifted results in a separation of cell replication from cell growth; i.e. population growth from individual growth. This would indicate that magnesium-limitation is primarily exerting its effect within the cell division cycle with the inference that magnesium-deficiency causes a blockage of cell division at the G_2/M boundary of the cell cycle (Walker and Duffus, 1980). From this, as has been argued by James (1961), care must be taken in defining growth; either on a per cell basis or on a dry weight basis.

Within the spike experiment, the patterns of cellular magnesium are similar to those within the pulse experiment although the actual cellular magnesium levels are significantly higher than for the pulse. The changes in the oxygen uptake rate are very similar to the pulse experiment but take place over a much shorter time-scale. However, the spike causes a lowering of ethanol production and glucose consumption on a per cell basis possibly reflecting a degree of synchrony in the cell cycle within the population. The findings that a magnesium shift-up of magnesium-limited cultures caused a synchronization of cell division within *S. pombe* (Walker and Duffus, 1980) are therefore relevant.

It is noted that at a dilution rate of 0.15hrs^{-1} the mean residence time of the cells is 6.7hrs. This coincides with the major changes occurring to the population parameters as a response to the pulse as well as being coincident with the maximum cellular

magnesium concentration within the pulse experiment.

Much of the data from other authors, referred to comparatively, are based on shake-flask studies that permit only a limited control of parameters thus rendering the data of limited value (van Dijken *et al.*, 1990; Fiechter *et al.*, 1981). Bioreactors are understood to permit a better control of physical parameters and thereby increase the reliability of the data but the limitations of batch studies still apply; i.e. the environmental conditions are changing continuously from the moment of inoculation and never fully stabilise making reproducibility and the ability to infer direct cause-and-effect extremely difficult (Fiechter *et al.*, 1981). Chemostats can introduce a particular regulatory (or regulated) state and maintain it within a constant environment.

Magnesium is known to be a bulk intracellular species with regard to both its total cellular level and its 'free' cytoplasmic levels (0.5-5% of the total). However, eukaryotic cells possess a lower total calcium content (3-5mM) and a lower 'free' calcium concentration (100-300nM), (Rasmussen, 1986). This implies that any uptake of calcium from the environment or release from intracellular stores will result in a more significant impact on the free calcium concentration (representing only 0.1% of the total). However, any alteration of magnesium's transmembrane flux is widely regarded as being insufficient to exert a controlling influence on cellular metabolism; however, this assumes that the regulatory action must be acute and rapid (Maguire, 1990). Hence, it is argued that changes will not be rapid, as for calcium, but will be relatively slow, in the order of minutes and hours and will be more sustained; this is referred to as "setting the gain or sensitivity of the responding system" as opposed to calcium's "on-off" effect (Maguire, 1990). The findings presented here support this argument.

Maguire (1990) focuses on the criterion to be met if a regulatory function for magnesium is to be inferred within higher eukaryotic cells and points out that the actual intracellular magnesium concentration is two orders of magnitude lower than predicted by theory that supports the conclusion that transport, cellular content, and intracellular levels are tightly regulated.

1) Cellular processes must exist which can respond to the physiological levels of free magnesium.

2) Transport mechanisms must exist which can effect changes in the intracellular free magnesium bearing in mind that: a) changes in pH will affect the binding of magnesium; b) there is no hard evidence for the release of magnesium from intracellular stores and internal buffering (Corkey *et al.*, 1986) reduces the possibility even further; c) transmembrane flux may change giving alterations in the overall magnesium content that may yield a change in the free magnesium levels through the buffering processes (Corkey *et al.*, 1986) with just small changes in overall content yielding significant changes in free magnesium (Maguire, 1990).

3) Compartments storing magnesium must be in communication with the responding cellular processes. Magnesium is compartmented in many types of eukaryotic cells (Grubbs, Collins, & Maguire, 1985; cited by Maguire, 1990) mostly in the cytosol but not all to the same extent (Maguire, 1990) and because most magnesium-sensitive processes are located within the plasma membrane then a communication mechanism must exist, although again there is no hard evidence yet (Maguire, 1990).

4) Changes in the intracellular free magnesium and the magnesium-sensitive process must occur concurrently, sequentially, and in an integrated fashion.

According to Maguire (1990), there is no specific event occurring within higher eukaryotic cells detailed in the literature that has a demonstrated requirement for magnesium as an activator. However, the initiation of cell division in *S. pombe* (Walker, 1986) is cited as being a possible candidate (Maguire, 1990) and is now extended by this work to include *S. cerevisiae*.

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Appendix 1

Gas Chromatography: Parameters for the separation and detection of ethanol from centrifuged fermentation samples.

Section 1: Conditions

			A	B
Oven Temperature	(^{o}C)	=	120	180
Isothermal Time	(min)	=	1.0	0.0
Ramp Rate	(^{o}C/min)	=	20.0	0.0
Injection Temperature	(^{o}C)	=	210	210
Detection Temperature	(^{o}C)	=	210	210
FID Sensitivity		=	Low	Low
FID Zero		=	On	On
Carrier Gas		=	N_2	N_2
Flow Rate	(ml/min)	=	45	45

Section 2: Data Handling

A) Data Acquisition

Start Time	=	0.00 min
End Time	=	4.00 min
Width	=	5
Area Sensitivity	=	96
Baseline Sensitivity	=	5
Skim Sensitivity	=	0
Baseline Correction	=	B-B

B) Report

Calculation Type = Internal Standard

Calculation From = Area

Printer Tolerance = 0.0000

Output Device = Screen

C) Peak Identification

Unretained Peak Time		=	0.00 min
Area/Height Reject		=	0.10
Reference Peak : Time		=	2.30 min
	: Tolerance	=	0.15 min
Component	: Tolerance Absolute	=	0.05
	: Tolerance Percentage	=	0.50

D) Quantitation

Scaling Factor	=	100
Standard Component Name	=	Iso-Propanol
Standard Component Amount	=	(weight of 0.025ml)
Sample Amount	=	(weight of 0.5 ml)

E) Component List

Retention Time	Response Factor	Standard Amount	Name
1.43	1.7221	(weight)	Ethanol
2.30	1.5610	(weight)	Iso-Propanol

Appendix 2

High Pressure Liquid Chromatography: Parameters for the separation and detection of sugars from centrifuged fermentation samples.

Section 1: Conditions

Column	=	Aminex HPX-87H
Column Temperature	=	25 ^{o}C
Eluant	=	0.012 N H_2SO_4
Eluant Flow-Rate	=	0.6 ml/min
Gradient	=	None used

Section 2: Data Handling

A) Data Acquisition

Start Time	=	6.67 min
End Time	=	20.0 min
Average Points	=	4
Sum Points	=	2

B) Report

Calculation Type = External Standard

Calculation From = Peak Area

Printer Tolerance = 0.0000

Output Device = Printer

C) Peak Integration

Smoothing Width	=	7
Initial Slope Threshold	=	100
Minimum Baseline Width	=	30
Initial Peak Width	=	30
Width Percentage Change	=	20
Shoulder Percentage Width	=	20
Minimum Peak Area	=	500

D) Peak Identification

Scaling Factor = 1

External Standard File = Sugars

E) Component List

Retention Time	Slope	Intercept	Name
6.79	4.06×10^{-6}	-2.34×10^{-1}	Raffinose
7.34	3.55×10^{-6}	-7.16×10^{-2}	Sucrose
8.62	3.17×10^{-6}	-5.30×10^{-4}	Glucose
9.44	3.20×10^{-6}	-2.25×10^{-4}	Fructose
12.45	3.97×10^{-6}	-2.76×10^{-3}	Glycerol
14.8	6.44×10^{-6}	-2.92×10^{-3}	Acetic Acid

Appendix 3

Atomic Absorption Spectrophotometer: Parameters for the detection of magnesium in centrifuged fermentation samples

Section 1: Conditions

Fuel	= Acetylene
Flow-rate	= 2.5 ml/min
Oxidant	= Air
Flow-rate	= 8.0 L/min
Lamp	= Ca/Mg lamp
Current	= 20 mA
Wavelength	= 285.2 nM
Slit	= 0.7 nM

Section 2: Data Handling

A) Data Acquisition

Integration Time	=	3 seconds
Replicates	=	3
Signal Processing	=	Hold
Technique	=	Atomic Absorption

B) Quantification

Reference to Linear Calibration Graph

Standards = 0.04/0.10/0.20/0.30/0.40/0.44/0.50 mg/L

Appendix 4

Deionization Procedure for Glassware and Plastics

1) Articles to be deionized were immersed in a 2%v/v nitric acid solution for at least eight hours.

2) The articles were then rinsed at least twice using ultra-pure water to remove any traces of the nitric solution.

3) The articles were then immersed in a solution of 0.1M EDTA for at least one hour.

4) Four separate rinses with ultra-pure water removed any traces of the EDTA solution.

5) Glassware was placed in a sterilizing oven at 160^{o}C until dry whereas plastic was placed in a 55^{o}C oven until dry.

6) Articles were used immediately upon drying to limit contamination from dust.

Appendix 5

Calculation Procedure for Magnesium Levels within the Medium

203.302g of $MgCl_2.6H_2O$ in 1 litre of H_2O gives a one molar solution of $MgCl_2.6H_2O$

20.33g of $MgCl_2.6H_2O$ in 1 litre of H_2O gives a 0.1 molar solution of $MgCl_2.6H_2O$

Accepting that one mole of $MgCl_2.6H_2O$ yields one mole of Mg then the stock solution will be a 0.1 molar solution of Mg; i.e. 100mM Mg.

Appropriate volumes of this stock solution added to known quantities of medium will yield the required magnesium levels.

Calculation Procedure for Converting ppm to mM Magnesium

One Mole of Mg	=	24.305g/L
One Mole of Mg	=	24305mg/L
4.114×10^{-5}M Mg	=	1mg/L
0.04114mM Mg	=	1mg/L
0.04114mM Mg	=	1ppm
mM Mg	=	ppm x 1/24.305